THE USE OF 3-DAY PERIODS IN HUMAN METABOLISM STUDIES [1]

CALCIUM AND PHOSPHORUS

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TWO FIGURES

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A number of studies of mineral metabolism during pregnancy have been carried out in this country and abroad. Macy et al. ('30), Coons et al. ('34) and Donelson ('31) have developed an excellent mechanical technic for making such studies in the home, and after extensive experimentation have selectd a 4- to 6-day balance period as representative and adequate for studying mineral retentions. They recommend, too, that continuous balance periods where food ingestion varies from day to day be grouped into these shorter segments in order to bring out both the individual's variability and her average physiological tendencies throughout the prenatal period.

In attempting to adapt their methods to our studies of prenatal metabolism while women were living under normal home conditions, we considered the possibility of using a shorter balance period than those recommended by them. Duties in the home are multiple and a long period is often such a burden to the mother that the benefits do not seem to

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warrant the time invested. We finally selected as a basis for experimentation a 24-hour period preceded by 2 consecutive days of preparation during which the food eaten was as identical qualitatively and quantitatively to the third, or collecting, day as the subject could make it within the limits of her appetite. Emphasis was put upon the need for similarity in preparing each food item eaten during the 3 days. This, of course, instituted a procedure unlike the ordinary habits of an adult since normally very few people eat exactly the same food prepared in exactly the same way for even 2 successive days. However, the need for approaching physiological stability seemed important if one were trying to get an idea of the metabolic state during pregnancy through intermittent sampling.

The object of this paper, then, is to present data gathered from the experiments so conducted and to compare them with the work done by Macy et al. ('30) and Coons et al. ('34) on the averaged 4- to 6-day periods in order to arrive at some conclusion as to variability, and to test the validity of 24-hour sampling periods which have been preceded by 2 days of preparation. Calcium and phosphorus metabolism have been considered first.

SUBJECTS

The subjects were all young married women between the ages of 22 and 34 years living in their own homes, except number II who was a married resident subject at Fels House. Forty-nine balances have been determined by Fels Fund workers using ten subjects. The four subjects whose balances are given in table 1 were selected for comparison with Macy's and Coons' subjects because they had no calcium or phosphorus therapy during pregnancy and their abnormalities seem slight enough to be called intermittent or temporary. Subjects II and IV were primiparae and gave birth to a girl and a boy respectively. Subjects VI, VII and VIII were secundiparae and gave birth to a girl, a boy, and a girl respectively. Infants numbers IV and VI may have been

slightly postmature; the other were called normal term infants. Their weights and physical characteristics will be described later. Subject number IV was found to be somewhat anemic during the fourth month in pregnancy, and at the suggestion of her physician ate more iron-containing foods thereafter to arrest this tendency. Number VI was thought

TABLE 1

Calcium and phosphorus utilization during pregnancy; 24-hour samplings expressed in grams per day

		CALCIUM				PHOSPHORUS			
Subject's number	Weeks pre-delivery	Food	Urinary	Fecal	Balance	Food	Urinary	Fecal	Balance
II	31	1.072	0.256	2.302	—1.486	1.116	0.732	1.523	—1.139
	27	1.058	0.288	2.146	—1.376	1.101	1.435	1.570	—1.904
	11*	1.073	0.372	1.439	—0.738	1.117	0.946	0.866	—0.695
	11*	1.103	0.291	1.277	—0.465	1.149	0.908	0.730	—0.489
	11*	1.104	0.356	1.397	—0.649	1.150	1.052	0.815	—0.717
IV	31	1.400	0.185	1.654	—0.439	1.461	0.848	0.807	—0.194
	27***	1.183	0.241	0.866	+0.076	1.410	0.863	0.615	—0.068
	23	1.266	0.214	1.318	—0.266	1.887	0.915	0.896	+0.076
	19***	1.721	0.260	1.168	+0.293	1.867	0.909	0.659	+0.299
	14	....**	0.212	1.684	....	....	1.230	0.759	....
	7***	1.351	0.213	0.832	+0.306	1.797	0.765	0.500	+0.532
	1***	2.078	0.138	1.594	+0.346	1.489	1.232	0.799	—0.542
VI	23	0.911	0.175	1.511	—0.775	0.885	0.634	0.880	—0.629
	19	1.756	0.183	1.303	+0.270	1.123	0.843	0.566	—0.286
	14***	1.255	0.210	0.898	+0.147	1.036	0.384	0.623	+0.029
	11***	1.591	0.152	1.056	+0.383	1.899	0.910	0.462	+0.527
	7***	1.725	0.252	1.403	+0.071	1.407	0.871	0.776	—0.240
	3	1.578	0.141	1.558	—0.121	1.390	0.686	0.898	—0.194
VII	11***	1.853	0.204	1.229	+0.420	1.397	0.707	0.777	—0.087
	7***	1.856	0.235	0.914	+0.707	1.939	0.858	0.589	+0.492
	1***	1.656	0.484	0.644	+0.528	1.543	0.708	0.375	+0.460
VIII	12***	2.076	0.366	1.400	+0.312	1.590	0.848	0.808	—0.066
	8***	1.009	0.357	1.002	—0.348	1.527	0.656	0.863	+0.008
	4	1.675	0.276	0.834	+0.567	1.414	0.356	0.574	+0.484
	1	1.875	0.214	0.970	+0.691	1.094	0.702	0.701	—0.309

* Three consecutive days.

** Sample lost.

*** Fels balances used in the matched comparisons. The other balances for each subject are included to show the general findings for these individuals.

to be anemic also, and usually suffered from hay fever during August which was her ninth month in this pregnancy. Neither condition was treated. Subject number VII had some albuminuria in the ninth month; number VIII had a persistent mild allergy toward egg and lemons and had suffered mildly from hay fever since childhood. The hay fever appeared a few days before delivery and she received treatment for it as usual.

PROCEDURE

The data were collected during the spring and summer of 1934 on subjects VI, VII and VIII, and on subject IV during the spring and summer of 1933. Periods were begun as a rule on Monday or Tuesday to insure completion during the same week. The women prepared and weighed their food in their own homes under our instruction. They were told to select foods which were representative of their usual meals and to eat, at the same time of day for 3 successive days, the same kind and as nearly the same amount of food as possible. On the third day the subject saved a day's sample of food equivalent either to the amount she ate or to one-half the amount. The feces were marked off with carbon and carmine given by capsule at the beginning of breakfast on the third and fourth days respectively. The women were asked to take a very small amount of water with the capsule. Tap water ingested throughout the day was weighed in grams and a similar quantity was included in the food sampling. A medical examination was made just before the period began and again when it ended, and basal metabolic rate and blood samplings were secured on the morning following the 3-day sampling period. The procedure outlined above was repeated every 28 days when possible, during the whole course of the prenatal period when the women were under observation.

METHODS USED IN ANALYSIS

Food, fecal, urinary, and blood samples collected for mineral analyses were all dried on the steam bath and then ashed

in the muffle furnace at a temperature below 400°C. until a loose, grayish ash was produced. The ash was put into solution with warmed, constant-boiling HCl and redistilled water. The residue was re-ashed and treated again to insure that the mineral was freed from carbon particles. Samplings from this solution made to volume were used for all analyses.

Calcium in food, feces and urine was determined volumetrically in triplicate, observing the precautions cited by Washburn and Shear ('32); phosphorus was determined by the method used by Embden and Fetter ('21).

METHOD OF COMPARISON

As stated in the beginning, the object in making this study was to see how data collected by the 24-hour method of sampling compared with the results presented by Coons ('30, '34) and by Macy ('30) and their co-workers on longer period samplings. Accordingly the following procedure was used. Twelve balances were selected from our data, and twelve each from the first and second studies made by Coons and her co-workers in order to match the twelve given by Macy and her co-workers. Balances determined consecutively on the same subject were used as far as was possible in order to fulfill two criteria for matching; namely, that the four sets of twelve balances should each fall in the same period in pregnancy, and should be representative of approximately the same number of individuals. The tables and the graphs show how close the matching could be made and how the results compare.[2]

[2] The Fels balances used are starred in table 1. Coons' first group ('30) is designated as Coons I and her second group ('34) as Coons II. In her first study (Coons I) all balances for subjects A and D, the third balance for subject B, the first and second balance for C, and the third balance for E were used in the matching. The balances selected from her second study (Coons II) were: the first and second for subject I, all for II, the first three for V, and the first and last two for VI. All of the balances given by Macy and her co-workers ('30) were used here.

From these forty-eight calcium and phosphorus balances taken from the four studies, comparisons were made in the following ways between each group of twelve balances:

a. Means, standard deviations, coefficients of variation and their probable errors for Macy, Coons and Fels balances were computed (table 2).

b. Urinary and fecal partitions for both calcium and phosphorus were calculated by dividing the excretion by the ingestion. Means, standard deviations and coefficients of variability were calculated for the partitions and included in table 2.

c. Pearson's product-moment correlations for food and fecal, and for food and urinary calcium and phosphorus were computed for each of the four groups of matched balances.

d. Diagrams were made showing the distribution of the balances around curves computed by the Method of Least Squares.

e. The findings in (a) and (b) were compared by computing critical ratios (table 3). Our basis for interpreting these ratios is given in the handbook of Chemistry and Physics, eighteenth edition, page 180. When the ratio was less than 2.0 the differences found were called insignificant; between 2.0 and 3.0, somewhat or tending toward significance; when the ratio was 3.0 or over, the difference was called significant.

The means, standard deviations and coefficients of variation in table 2 are given only to clarify the critical ratios. It is obvious that variability should be high for average mineral output in feces and urine because the balances are scattered throughout most of the period of pregnancy. An average for excretion, therefore, computed from consecutive balances which are inevitably modified by rapid growth of the fetus and by the maternal needs is of little use except in matched comparison and where average variation is being considered. The averages for mineral content of the food, however, do show how uniform was the amount of calcium and phosphorus ingested by each of the four groups at various stages in pregnancy.

TABLE 2

Means, standard deviations and coefficients of variation for matched ingestion and excretion values

		MACY	COONS I	COONS II	FELS
Urinary calcium	M[1]	0.447±0.031	0.204±0.017	0.204± 0.021	0.215±0.016
	SD	0.162±0.022	0.087±0.012	0.104± 0.014	0.083±0.011
	C of V	33.9 ±5.19	42.6 ±6.85	60.0 ±10.8	38.6 ±6.05
Fecal calcium	M	1.549±0.092	0.857±0.049	1.034± 0.081	1.076±0.045
	SD	0.474±0.065	0.252±0.035	0.414± 0.057	0.233±0.032
	C of V	30.6 ±4.60	29.4 ±4.38	40.0 ± 6.33	21.7 ±3.13
Food calcium	M	1.922±0.065	1.149±0.058	1.537± 0.092	1.610±0.065
	SD	0.333±0.046	0.299±0.041	0.473± 0.065	0.335±0.046
	C of V	17.3 ±2.45	26.0 ±3.81	30.8 ± 4.63	20.8 ±2.99
Urinary calcium partition	M	0.253±0.017	0.182±0.016	0.128± 0.011	0.170±0.016
	SD	0.085±0.012	0.083±0.011	0.056± 0.008	0.084±0.012
	C of V	33.6 ±5.12	45.6 ±7.47	43.7 ± 7.07	49.4 ±8.30
Fecal calcium partition	M	0.797±0.049	0.736±0.027	0.660± 0.021	0.685±0.028
	SD	0.253±0.035	0.141±0.019	0.109± 0.015	0.144±0.020
	C of V	31.7 ±4.78	19.2 ±2.74	16.5 ± 2.33	21.0 ±3.01
Urinary phosphorus	M	1.241±0.053	0.723±0.024	0.744± 0.033	0.809±0.041
	SD	0.272±0.037	0.124±0.017	0.168± 0.023	0.210±0.029
	C of V	21.9 ±3.16	17.2 ±2.44	21.7 ± 3.13	26.0 ±3.81
Fecal phosphorus	M	0.747±0.037	0.580±0.034	0.59 ± 0.04	0.654±0.029
	SD	0.190±0.026	0.177±0.024	0.19 ± 0.03	0.149±0.021
	C of V	25.4 ±3.72	30.5 ±4.57	32.2 ± 4.87	22.8 ±3.30
Food phosphorus	M	2.248±0.081	1.442±0.063	1.74 ± 0.09	1.575±0.049
	SD	0.415±0.057	0.325±0.045	0.47 ± 0.07	0.252±0.032
	C of V	18.5 ±2.63	22.5 ±3.25	27.0 ± 3.98	16.0 ±2.26
Urinary phosphorus partition	M	0.555±0.014	0.527±0.014	0.452± 0.009	0.516±0.023
	SD	0.070±0.010	0.073±0.010	0.047± 0.006	0.117±0.016
	C of V	12.6 ±1.76	13.9 ±1.95	10.4 ± 1.45	22.7 ±3.28
Fecal phosphorus partition	M	0.575±0.035	0.403±0.017	0.337± 0.015	0.397±0.029
	SD	0.181±0.025	0.087±0.012	0.079± 0.011	0.147±0.020
	C of V	31.5 ±4.75	21.6 ±3.11	23.4 ± 3.39	37.0 ±5.75

[1] In this table M indicates mean, SD indicates standard deviation, and C of V indicates coefficient of variation.

TABLE 3

Critical ratios

	MACY AND COONS I	MACY AND COONS II	COONS I AND COONS II	MACY AND FELS	COONS I AND FELS	COONS II AND FELS
Urinary calcium						
M[1]	6.9 (M)[2]	6.6 (M)	0	5.3 (M)	2.2 (F)	2.0 (F)
SD	3.1 (M)	3.1 (M)	0.9 (II)	2.6 (M)	0.4 (F)	0.5 (C)
C of V	1.0 (C)	2.2 (C)	1.4 (II)	0.3 (F)	0.7 (C)	1.9 (C)
Fecal calcium						
M	6.6 (M)	4.2 (M)	1.9 (II)	4.6 (M)	3.3 (F)	0.5 (F)
SD	3.0 (M)	0.7 (M)	2.4 (II)	3.3 (M)	0.4 (C)	2.4 (C)
C of V	2.0 (C)	1.2 (C)	1.7 (II)	1.6 (M)	1.4 (C)	2.8 (C)
Food calcium						
M	8.9 (M)	3.4 (M)	3.7 (II)	3.4 (M)	5.3 (F)	0.9 (F)
SD	0.6 (M)	1.8 (C)	2.3 (II)	0.0 (F)	0.6 (F)	1.7 (C)
C of V	2.0 (C)	2.6 (C)	0.8 (II)	0.9 (F)	1.2 (C)	1.8 (C)
Urinary calcium partition						
M	3.0 (M)	6.2 (M)	2.8 (I)	3.6 (M)	0.5 (C)	2.2 (F)
SD	0.1 (M)	2.0 (M)	2.0 (I)	0.1 (M)	0.1 (F)	1.9 (F)
C of V	1.3 (C)	1.2 (C)	0.2 (I)	1.6 (F)	0.3 (F)	0.5 (F)
Fecal calcium partition						
M	1.1 (M)	2.6 (M)	2.2 (I)	2.0 (M)	1.3 (C)	0.7 (F)
SD	2.7 (M)	3.8 (M)	1.3 (I)	2.7 (M)	0.1 (F)	1.4 (F)
C of V	2.5 (M)	2.9 (M)	0.8 (I)	1.9 (M)	0.4 (F)	1.2 (F)
Urinary phosphorus						
M	8.9 (M)	7.5 (M)	1.3 (II)	6.5 (M)	1.9 (F)	0.7 (F)
SD	3.6 (M)	2.4 (M)	1.5 (II)	1.3 (M)	2.6 (F)	1.1 (F)
C of V	1.2 (M)	0.0 (M)	1.1 (II)	0.8 (F)	1.9 (F)	0.9 (F)
Fecal phosphorus						
M	3.3 (M)	2.9 (M)	0.2 (II)	1.9 (M)	1.7 (F)	1.4 (F)
SD	0.4 (M)	0	0.4 (II)	1.2 (M)	0.9 (C)	1.2 (C)
C of V	0.9 (C)	1.1 (C)	0.3 (II)	0.5 (M)	1.4 (C)	1.6 (C)
Food phosphorus						
M	7.9 (M)	4.2 (M)	2.7 (II)	7.1 (F)	1.7 (F)	1.6 (C)
SD	1.3 (M)	0.6 (C)	1.8 (II)	2.5 (M)	1.5 (C)	2.8 (C)
C of V	0.9 (C)	1.8 (C)	0.9 (II)	0.7 (M)	1.6 (C)	2.4 (C)
Urinary phosphorus partition						
M	1.4 (M)	6.2 (M)	4.5 (I)	1.4 (M)	0.4 (C)	2.6 (F)
SD	0.2 (C)	2.0 (M)	2.2 (I)	2.5 (F)	2.3 (F)	4.1 (F)
C of V	0.5 (C)	1.0 (M)	1.4 (I)	2.7 (F)	2.3 (F)	3.4 (F)
Fecal phosphorus partition						
M	4.4 (M)	6.3 (M)	2.9 (I)	3.9 (M)	0.2 (C)	1.8 (F)
SD	3.4 (M)	3.7 (M)	0.5 (I)	1.1 (M)	2.6 (F)	3.0 (F)
C of V	1.7 (M)	1.4 (M)	0.4 (II)	0.7 (F)	2.4 (F)	2.0 (F)

[1] M indicates mean, SD indicates standard deviation, and C of V indicates coefficient of variation.

[2] In this table the letter in parenthesis indicates which study had the higher value. For example, 6.9 (M) indicates that Macy's group had the higher value in addition to being of significantly different value than Coons I.

CALCIUM

Ingestion. The figures for average ingestion and excretion are given in table 2, and for the significance of any difference in table 3. Macy's group had the greatest average calcium ingestion, Fels group next, Coons II next and Coons I the least in amount. The critical ratio showed that each of them received a significantly different amount except when Coons II and the Fels group were compared. Although Macy's subjects varied less than the other three in average calcium ingestion, the differences in variability were not significant except when her results were compared with Coons II. The latter group was about 13.5 per cent more variable than the former.

Partition. A number of significant differences appeared when the average calcium partition was calculated. Partition as used here means the figure obtained by dividing the amount of calcium excreted in urine and feces respectively by the amount contained in the food. Means, standard deviations and coefficients of variability were calculated for partitions in each of the four studies. These results were examined with particular interest, because it was felt that there the effects of mineral 'lags' might be revealed if the partitions for the Fels Fund study were decidedly atypical with respect to other values computed for pregnancy.

Possibly because the ingestion values were greater, the average urinary calcium partition for Macy's group was greater than the other three groups. The figure was not significantly more variable than the other three. Coons I, with the next highest value, differs significantly from Macy's group and tends to have a greater partition figure than Coons II. Coons I has a somewhat greater standard deviation, but the variability in Coons' two studies is very similar.

The Fels group ranks between Coons I and Coons II in average urinary calcium partition. In the Macy-Fels pairing, Fels subjects were more variable on the average, and the Coons II-Fels pairing showed that the Fels group had the greater standard deviation. However, the critical ratios for the standard deviations of the latter pairing is 1.9, and for

Coons II-Macy and Coons II-Coons I pairings are each 2.0. Consequently the urinary calcium partition in the Fels group does not seem to be clearly aberrant.

Fecal partition should be influenced by mechanical defects in marking. There is no doubt that this hazard is great in 24-hour sampling. However, the calculations revealed no significant differences in the four studies. Macy's group and Coons II differ most in mean, standard deviation, and coefficient of variation. Coons I and Coons II tend to differ in the mean. Fels' and Macy's groups differ in mean and standard deviation and that difference approaches significance. The Fels group and Coons I and Coons II are quite similar in all respects. On the whole Coons II and Macy's groups differ the most in fecal partition.

PHOSPHORUS

Ingestion. Again the means, standard deviations, coefficients of variation and partitions given in table 2 were studied, and the critical ratios given in table 3 were examined to see whether the groups differed significantly. Macy's group had ingested the most phosphorus on the average. Matched against Coons I and II and the Fels group, this difference in average phosphorus ingestion becomes significant, but the amount ingested is not more variable. Coons II ranks next highest, and those subjects tended to use more variable quantities than the Fels group, which had the third greatest phosphorus ingestion. Coons I had the smallest phosphorus ingestion and tended to differ significantly from Coons II. Coons I and the Fels group are more alike in the quantity of phosphorus ingested than any other paired groups.

Partition. Urinary phosphorus excretion per gram of phosphorus intake shows a higher mean value for Macy's group than for the other three. The difference is not significant except where her findings are paired with Coons II. Macy's average partition resembles Coons I, but there is enough difference in means between Coons I and Coons II

and between Macy's group and Coons II to permit one to conclude that there is as much difference in the average for urinary phosphorus partition in these three studies as there is between any one of them and the Fels 24-hour period samplings judged from the balances selected here. Macy and Coons II tend to differ in standard deviation, but the difference between them in variability is negligible. Fels mean value ranks between the figures for Coons I and II, and has a significantly greater standard deviation and variability than all three of the other groups. The Fels mean tends to differ significantly only when matched against Coons II.

The calculation for fecal phosphorus output per gram of phosphorus intake gives Macy's group the highest value on the average. Her group partition again differs significantly from the other three. The standard deviation is significantly greater than Coons I and Coons II and is slightly greater than the value for Fels group. Coons I differs from Coons II in the mean, but the variability is similar and the range of variability in Coons' subjects is less than Macy and Fels Fund groups.

DISCUSSION[8]

A clear-cut difference between the long and short sampling period for calcium and phosphorus metabolism during pregnancy was not found when the four studies were compared by matching averages, distribution, and range in mineral

[8] Only one subject, subject II (see table 1), has been observed on each day of the 3-day balance period. She had just recovered from a rather severe cold, contracted while taking a long automobile trip during a brief holiday. When she returned, she was asked to eat the same kind of food which she had eaten in December and January and to eat as near the same amount of food for 3 days as she could. It was interesting to note that she was continuously in negative balance. At the beginning of pregnancy she was in poor physical condition as her food intake had been very much restricted. Yet after 4 months of quite intelligent and abundant choice of fresh fruits, vegetables and meat and the use of a quart of milk, cod liver oil and yeast daily, she was still in negative balance. Her gain in weight had been rapid and she had been active and well except for the one cold. The results for the 3 successive days differ, but interestingly enough, either the first day or the third day approaches the average of the 3 days fairly closely in calcium or phosphorus distribution. Calculations for differences on each of the 3 days on other subjects are in progress.

balance figures. For phosphorus the results are less clear-cut than for calcium, but the general evidence seemed to us to point toward differences due to differences in the individuals who were studied rather than to the mechanics of sampling. A further test was applied by examining the correlations made to show the relation between ingestion and excretion for both calcium and phosphorus (see table 4).

TABLE 4

ITEMS CORRELATED	OBSERVER	r	PE_r
Food and fecal calcium	Macy	+0.02	±0.20
Food and fecal calcium	Coons I	+0.84	±0.06
Food and fecal calcium	Coons II	+0.95	±0.02
Food and fecal calcium	Fels	+0.41	±0.16
Food and urinary calcium	Macy	+0.04	±0.19
Food and urinary calcium	Coons I	+0.17	±0.19
Food and urinary calcium	Coons II	+0.01	±0.19
Food and urinary calcium	Fels	—0.03	±0.19
Food and fecal phosphorus	Macy	+0.35	±0.17
Food and fecal phosphorus	Coons I	+0.66	±0.11
Food and fecal phosphorus	Coons II	+0.83	±0.06
Food and fecal phosphorus	Fels	—0.33	±0.17
Food and urinary phosphorus	Macy	+0.55	±0.16
Food and urinary phosphorus	Coons I	+0.86	±0.05
Food and urinary phosphorus	Coons II	+0.92	±0.03
Food and urinary phosphorus	Fels	—0.42	±0.16

Some factor tends to shift the Fels results toward a species of negative relationship—the greater the ingestion, the greater the retention, although the correlation is too low to be used as conclusive evidence. Correlations made on Macy's calcium findings are equivocal, and the correlations for phosphorus are too low to be conclusive. The indication is that the greater the phosphorus ingestion, the greater might be the excretion. On the other hand, Coons' results are quite clearly positive for phosphorus, namely, the greater the ingestion, the greater the excretion, but the results were equivocal for urinary-food calcium correlation. This is interesting and puzzling since Macy's group received the largest amounts of both calcium and phosphorus on the average, and calcium

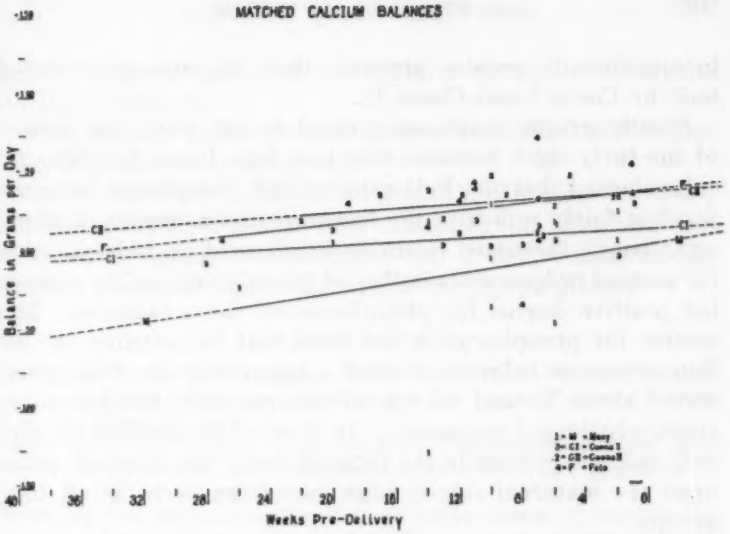

Figure 1

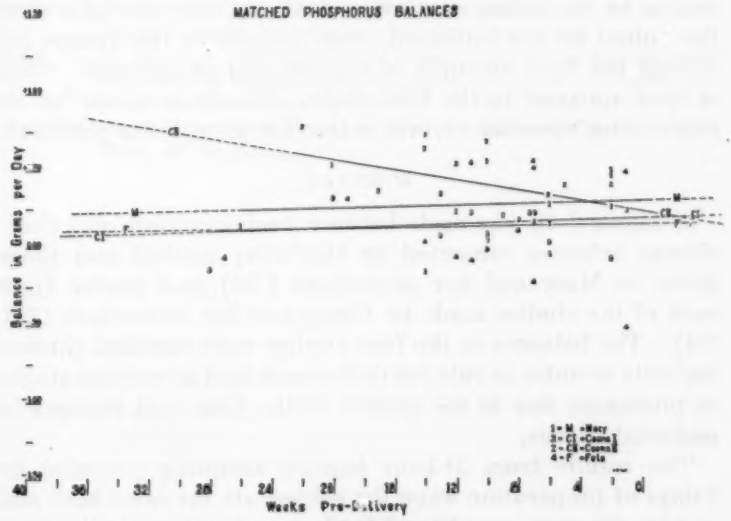

Figure 2

These charts show the distribution and average trends of the four groups of balances discussed in the paper. The curves were computed by the method of least squares.

in significantly greater amounts than the amount received both by Coons I and Coons II.

Finally graphs were constructed to see what the scatter of the forty-eight balances was (see figs. 1 and 2). The results showed that the Fels calcium and phosphorus balances fitted in fairly well with the longer period averages of Macy and Coons. Increased retention which must be a logical state for normal pregnancy is indicated for calcium, and in a lesser but positive degree for phosphorus by these balances. The scatter for phosphorus is less than that for calcium for all four groups of balances charted. Apparently the Fels group stored about 7.0 and 4.5 gm. of calcium and phosphorus respectively during pregnancy. In view of the amount of mineral which is present in the fetus at birth, the demands made upon the maternal stores must have been large in all four groups.

It was interesting to note that the figure for calcium excretion by the kidney route was decidedly more variable than the output by the intestinal route in each of the groups receiving the least amounts of calcium and phosphorus. This is most apparent in the Fels study. We are at a loss for an explanation since the reverse is true for phosphorus partition.

SUMMARY

A comparison was made between twelve calcium and phosphorus balances computed by the 3-day method and those given by Macy and her co-workers ('30), and twelve from each of the studies made by Coons and her co-workers ('30, '34). The balances in the four studies were matched chronologically in order to rule out differences seen at various stages in pregnancy due to the growth of the fetus and changes in maternal tissues.

The results from 24-hour balance sampling preceded by 2 days of preparation when the subject ate the same kind and as near the same quantity of food as on the third (collecting) day did not differ significantly enough from the results given in the other three studies to indicate that a period of this

length fails to show an individual's metabolic tendencies during pregnancy.

The findings point toward greater individual differences than difference due to method.

On the strength of these indications we feel that the 3-day balance period with a 24-hour sampling on the third day merits consideration as a clinical tool in determining human calcium and phosphorus utilization during pregnancy.

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THE POTENTIAL ALKALINITY OF HONEY: ITS ACID-BASE VALUE AS A FOOD

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No very extensive study of the mineral components of honey has been made, although a number of investigators have carried out limited studies of the composition of the ash of various types of honey. These investigations, however, were mostly of foreign honeys, so that there is actually very little information relative to the composition of American honeys from this standpoint.

The following mineral elements have been found in honey: Phosphorus (Elser, '25, '26), calcium (Elser, '28; Stitz, '27), potassium (Svoboda, '33), magnesium (Jewell, '31), sodium (Nottbohm, '27), chlorine (Nottbohm, '14), sulphur (Nottbohm and Weinhausen, '14), manganese (Gottfried, '11, '12), iron (Schuette and Remy, '32), aluminum (Jewell, '31), copper (Lindow, Elvehjem and Peterson, '29), and silicon (Jewell, '31). The relative proportions in which these elements occur appear to vary widely, although in general phosphorus, calcium and potassium predominate.

Nutrition specialists consider honey almost altogether as an energy food, as it contains usually from 75 to 80 per cent of sugars. Since the average ash content is quite low, and only comparatively small amounts of honey can be eaten at one time, the mineral elements have been given very little consideration. However, increasing quantities of honey are now being used for confectionery purposes and in baking, particularly bread, so that much greater quantities may be consumed by an individual in this way.

Although it has been shown that honey yields a decidedly alkaline ash (Nelson and Mottern, '31), the acid-base balance of honey as a food has not been studied. When the alkalinity of the ash is used as a measure of the acid-base balance, correction should be made for the loss of chlorine and sulphur which occurs during ashing. These losses have been pointed out by Nottbohm ('14).

Since the data on the composition of the mineral components of American honeys are too limited to permit calculations of the acid-base balance, the recently published method of Davidson and LeClerc ('35) for determination of the acid-base balance of foods offered a convenient means of obtaining these values for honey. Accordingly, this method was applied to the determination of the acid-base balance of a number of American honeys, including the more representative floral varieties.

Briefly, the method used consists of direct titration of the ash, with corrections for the sulfur and the chlorine lost during combustion. This procedure entails fewer and simpler determinations in comparison with the method based on computation from mineral analyses. Results are approximately the same by either method. Table 1 gives a summary of the results obtained. All the honeys studied gave definite alkaline values. With a few exceptions, values for the dark-colored honeys are somewhat greater than for the lighter-colored varieties, due apparently to the generally higher ash content of the darker types. The alkaline values closely parallel the ash contents. Honey is similar to fruits in that while definitely acid to the taste, due to the presence of free organic acids, as a food it is potentially alkaline. Apparently a considerable part of the metal elements present is combined with organic acid radicals (Nelson and Mottern, '31).

It might be of interest to compare the potential alkalinity of honey with some of the typically alkaline foods as given by Sherman ('32). While the average ash content of honey is comparatively low, the potential alkalinity compares favorably with some of the fruits and vegetables. For example, the average alkaline value for honey is 1.5, while that

for apples is 3.7; for asparagus, 0.8; for lemons, 5.0; for mushrooms, 4.0; for orange juice, 4.5; for potatoes, 7.0; for pumpkin, 1.5; for tomatoes, 5.6; for peas, 3.6; and for turnips, 2.7.

TABLE 1

Potential alkalinity of honeys of a variety of floral types

PREDOMINANT FLORAL SOURCE	COLOR GRADE (PFUND COLOR SCALE)		ASH CONTENT	TITRATION OF ASH (CC. N/10 ACID PER 100 GM. HONEY)	CORRECTION FOR S LOST IN COMBUSTION (CC. N/10 ACID PER 100 GM. HONEY)	CORRECTION FOR Cl LOST IN COMBUSTION (CC. N/10 ACID PER 100 GM. HONEY)	POTENTIAL ALKALINITY (CC. 1 N ALKALI PER 100 GM. HONEY)
	Reading [1]	Classification					
			per cent				
Sweet clover	0.6	Water white	0.04	4.50	None	1.80	0.27
Orange	1.2	Extra white	0.05	6.40	None	1.40	0.50
White clover	3.0	White	0.08	8.60	None	2.00	0.66
Sage	3.1	White	0.07	7.50	None	1.80	0.57
Tupelo	4.0	Extra light amber	0.09	10.25	None	1.90	0.84
Mesquite	4.5	Extra light amber	0.61	52.40	None	20.20	3.22
Catsclaw	8.5	Amber	0.22	21.80	0.43	2.80	1.86
Goldenrod	8.5	Amber	0.16	12.10	0.12	1.50	1.05
Tulip-Poplar	11.5	Dark	0.30	28.70	None	1.90	2.68
Mixed flowers	12.0	Dark	0.51	50.00	3.31	1.00	4.57
Buckwheat	13.5	Dark	0.10	8.20	0.90	3.10	0.42
Averages							
Light honeys [2]	...	.....	0.16				1.01
Dark honeys	...	.....	0.26				2.12
All honeys	...	.....	0.20				1.51

[1] Intensity of color increases with the numerical values (U. S. D. A., '33).

[2] First six samples are classified as light-colored honeys.

SUMMARY

The Davidson-LeClerc method for determining the acid-base value of a food by titrating the ash was applied to eleven samples of honey representative of both the light and dark varieties. The average alkaline value proved to be 1.5. While this is low, it compares favorably with certain fruits and vegetables, values of which are also given for the purpose of comparison.

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AN IMPROVED SYNTHETIC RATION FOR VITAMIN B_4 STUDIES [1]

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ONE FIGURE

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The importance of vitamin B_4 as a necessary constituent of the diet is rapidly gaining recognition, and accurate methods for assay of this factor are greatly needed. In a previous publication (Keenan, Kline, Elvehjem and Hart, '33) we described a paralysis which develops in chicks on diets low in vitamin B_4. A ration for assay and further study of the vitamin was proposed which in our preliminary studies was found quite satisfactory. The chick has been used extensively in this work, as well as for studies of other factors in the vitamin B complex, because of its well-known sensitivity to a lack of the water soluble dietary factors. The chick paralysis occurring on the synthetic diet which we have used has been prevented by feeding vitamin B_4 concentrates prepared according to the procedure proposed by Kinnersley et al. ('33) and by Barnes, O'Brien and Reader ('32). This successful use of the Reader concentrate established the identity of the rat deficiency disease (Reader, '29) with that occurring in chicks.

Extensive use of the synthetic diet described in the previous paper revealed seasonal variations in the response of the chicks receiving the basal diet, as well as those fed standard

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supplements containing the vitamin. These seasonal differences were thought to be due to variations in the storage of the factor in the chicks at hatching. However, recent work has shown that the response is also dependent to a large extent upon the vitamin B_4 content of the various constituents of the synthetic diet. Although the constituents were purchased in fairly large quantities, one or more of the products varied quite frequently.

In this paper we wish to report the results obtained when the synthetic diet was modified to reduce vitamin B_4 intake, as well as to increase certain other essential factors, and to describe a procedure for vitamin B_4 studies in chicks which we have found fairly successful.

EXPERIMENTAL

Methods of handling the experimental animals have been described in previous reports (Kline et al., '32; Keenan et al., '33). White Leghorn chicks, day old, were used exclusively in these studies and were obtained from the poultry department or from commercial hatcheries. In the earlier studies groups of ten animals were used, but when testing only slight modifications in the ration, three to four chicks have been used in each group.

Basal ration 441, the ration previously described, was found satisfactory, paralysis occurring at the fourth week in 100 per cent of the animals, until the summer months of 1934. The constitution of ration 441 is given in table 1. The constituents, undoubtedly, varied considerably in the amounts of water-soluble factors carried as contaminants. The crude casein was an acid-precipitated, domestic casein of good grade. Dextrin was made by autoclaving cornstarch for 2 hours, although by this process not all the starch is dextrinized. The salt mixture used was the Steenbock-Nelson ('23) salts 40, modified to contain sufficient amounts of copper, manganese and zinc. Eight per cent Anheuser-Busch dried yeast (strain C) was used in the diet, a level double that required to furnish sufficient vitamins $B(B_1)$ and $G(B_2)$ as shown by previous

TABLE 1

Composition of rations

CONSTITUENTS	R. 441	441A	441H	443	445	445H	446	448	449	450	451	452
Dextrin	53.5	53.5	53.5	57.5	57.5		70.5	71	70	69	64	64
Dextrin—heated 120°C.—24 hours						57.5						
Crude casein	24						12					
Crude casein—autoclaved 5 hours		24		24								
Crude casein—heated 120°C. —24 hours			24									
Crude casein—autoclaved and heated					24	24						
Casein—purified								18	18	18	18	18
Salts 40	2.5	2.5	2.5	2.5	2.5	2.5	2.5					
Salts I								5	5	5	5	5
A. B. yeast	8	8	8	8	8	8	8					
Brewers' yeast									1	2	2	2
Autoclaved liver residue	10	10	10				5	2	2	2	2	2
Autoclaved extracted liver residue				7	7							
Autoclaved extracted heated liver residue						7						
Crude liver extract powder								2	2	2	2	2
B_1 concentrate								5 γ daily				
Lung tissue											5	
H_2O extracted lung tissue												5
Cod liver oil	2	2	2	1	1	1	2	2	2	2	2	2

assay. The vitamin B_4 content of this yeast was very low, since feeding 30 per cent in the synthetic diet did not reduce the occurrence of paralysis in chicks. The 'growth factor' in liver, recently described (Kline et al., '34), was furnished in 10 per cent liver residue that had been autoclaved to destroy any vitamin B_4 remaining after water extraction. Vitamins A and D were supplied in cod liver oil.

During the summer months of 1934, the occurrence of B_4 deficiency in chicks receiving ration 441 was considerably decreased, and the rate of growth approached that of the positive control group which received vitamin B_4. The data in table 2 will show that of thirty animals receiving ration 441, eight failed to exhibit symptoms of the deficiency, while only two died of paralysis before the end of the 6 weeks experimental period. The average weight of these birds, 212 gm., indicated only slight deficiency of the vitamin. This growth is compared in figure 1 with that of a group receiving a vitamin B_4 supplement (18 per cent vacuum desiccated whole liver) in which growth was normal.

With this failure of ration 441 to allow occurrence of paralysis in 100 per cent of the animals, we attempted to modify the ration by heat treatment. In a study of the heat stability of the B complex factors (Keenan et al., '35) it was found that B_4 may be completely destroyed in liver by autoclaving for 5 hours at 120°C., and partially destroyed in a grain diet by heating in a dry oven at 120°C. 24 hours. Casein was considered the most likely carrier of vitamin B_4 as an impurity, since it is a good adsorbing agent for the water-soluble factors of milk. The casein was autoclaved for 5 hours at 120°C., then used in synthetic diet 441A (table 1). This proved an adequate modification for a short time, but over a long period the results were unsatisfactory, and in table 2 the data indicate that 45 per cent of the sixty-six birds which were fed ration 441A developed no deficiency symptoms during the 6 weeks experimental period. The average weight on this diet was 244 gm., a growth greater than that obtained on ration 441.

Treatment of the crude casein by heating dry in an electric oven at 120°C. for 24 hours, and fed in ration 441H (table 1), gave similar results with reference to occurrence of paralysis and rate of growth (table 2).

TABLE 2

Comparison of growth and occurrence of paralysis on synthetic diets

RATION	NUMBER OF CHICKS	AVERAGE WEIGHT 3 WEEKS, GRAMS	AVERAGE WEIGHT 6 WEEKS GRAMS	NUMBER DEAD AT 6 WEEKS	NUMBER SHOWING DEFICIENCY	NUMBER NOT SHOWING DEFICIENCY	SEVERITY OF GIBLED LESIONS
R. 441 only	30	95	212	2	22	8	—
R. 441 + B_4 supplement	30	134	360	0	0	30	—
R. 441A only	66	102	244	19	36	30	—
R. 441A + B_4 supplement	12	144	378	2	2	10	—
R. 441H only	3	98	200	0	3	0	—
R. 443 only	26	100	217	9	20	6	—
R. 443 + B_4 supplement	17	117	270	4	6	11	—
R. 445 only	5	70	105	0	3	2	—
R. 445 + B_4 supplement	9	122	237	0	1	8	—
R. 445H only	15	66	108	3	8	7	—
R. 445H + B_4 supplement	9	92	250	0	2	7	—
R. 446 only	9	55	74	2	7	2	—
R. 446 + B_4 supplement	12	75	160	1	5	7	—
R. 448 only	13	65	60	11	13	0	Severe
R. 448 + B_4 supplement	12	97	190	1	12 (slight)	0	Variable
R. 449 only	10	74	—	10	10	0	Marked
R. 449 + B_4 supplement	3	117	320	0	1	2	Marked
R. 450 only	12	66	—	12	9	3	Marked
R. 450 + B_4 supplement	6	120	375	0	0	6	Variable
R. 451 only	12	80	190	6	12	6	Slight
R. 451 + B_4 supplement	12	160	390	0	0	12	Normal
R. 452 only	26	78	—	26	26	0	Slight
R. 452 + B_4 supplement	15	147	360	0	0	15	Normal

Further purification of the liver residue was thought advisable. Since the 'growth factor' has been shown to be completely insoluble in water, additional water extraction of the liver residue, with removal of 30 per cent solids, had no

effect on its growth factor potency. This re-extracted residue was then autoclaved 10 hours, and fed in synthetic diet 443 (table 1) in combination with autoclaved crude casein. Again, reference to table 2 will show that this change did not reduce the rate of growth or increase the occurrence of paralysis. The record for the vitamin B_4 supplement used in ration 443 (table 2) indicates that B_4 had not been reduced materially, but that possibly other factors had been injured.

In synthetic ration 445, crude casein, which had been both autoclaved 5 hours and heated 24 hours at 120°C., was fed in combination with re-extracted autoclaved liver residue. Although the rate of growth of birds receiving this diet was markedly reduced, symptoms of B_4 deficiency were not observed in all cases. When vitamin B_4 was added to diet 445 growth was sub-normal. If, in addition to the autoclaved casein, the re-extracted liver residue and dextrin were subjected to heating at 120°C. for 24 hours (ration 445H, table 1), results similar to those with ration 445 were obtained.

With failure of heat treatment as a means of purification of the synthetic diet with respect to vitamin B_4, an attempt was made to modify the ration by reducing the crude casein and autoclaved liver residue constituents to 12 per cent and 5 per cent respectively. It seemed possible that essential nutritional factors, as yet undescribed, were present in the crude casein and liver residue, and were being destroyed by the drastic heat treatment used in diet 445H. We thought it possible to reduce the absolute amount of B_4 in the diet by reducing the casein and liver residue content, and still retain a supply of unknown constituents. Reference to table 2 will indicate that ration 446 (table 1) was unsuccessful with respect to rate of growth and occurrence of paralysis. Little more than maintenance of weight occurred in birds receiving this diet, and the onset of the paralytic symptoms was gradual, giving a chronic type of the deficiency disease. With the addition of 18 per cent whole liver as a B_4 supplement to ration 446, the growth rate was subnormal and paralysis occurred in several of the animals. A comparison is made in the growth charts for these animals presented in figure 1.

A source of uniform chicks. Throughout this work the lack of uniformity among individual chicks was a marked feature, particularly a lack of uniform response in groups of chicks of different hatching, and of different seasons of the year. Storage of nutritional factors in the egg is known to be influenced by the diet of the hen, which varies considerably during the year under ordinary poultry practices. To reduce this variation to a minimum, eggs were selected from a pen of twenty-four laying hens kept at the poultry department under the same conditions throughout the year. These hens were kept inside the brooder house with windows open, and were fed the following ration:

Basal mash

Ground yellow corn	100
Ground whole oats	50
Wheat bran	100
St. wheat middlings	100
Meat scrap	75
Dried skim milk	25
Ground alfalfa hay	50
Salt	5

Ration for hens

	Per cent
Basal mash	39.6
Cod liver oil	0.4
Whole yellow corn	40.0
Whole wheat	20.0

The hens kept under winter conditions throughout the year, and fed a ration relatively low in vitamin B_4, produced chicks with a uniformly low storage of this factor. These chicks were used in all subsequent experiments.

Further purification of the synthetic ration. The ideal synthetic diet is one in which the 'non-purifiable' constituents are reduced to a minimum; however, the cost of vitamin concentrates available is prohibitive for routine assay of a factor such as B_4. Synthetic diet 448 (table 1) was devised with the idea of reducing as much as possible the supplements containing the vitamin B factors, particularly.

In ration 448 the 8 per cent Anheuser-Busch yeast was supplanted by Merck crystalline vitamin $B(B_1)$, 5 micrograms daily, and 2 per cent liver extract powder as a source of vitamin G and flavin (Elvehjem and Koehn, '35). The growth factor supplement, autoclaved liver residue, was reduced to

a level of 2 per cent. Vitamins A and D were furnished in 2 per cent cod liver oil. At this point in our experiments it was found necessary, due to the fine sensitivity of the chick to inorganic salt balance, to replace salts 40 with salts I of the following composition:

Salts I

$NaCl$	335	$Fe(C_6H_5O_7)_2.6H_2O$	55
$K_2HPO_4.3H_2O$	845	KI	1.6
$Ca_3H_4(PO_4)_2.4H_2O$	190	$MnSO_4.4H_2O$	0.7
$MgSO_4.7H_2O$	204	$ZnCl_2$	0.5
$CaCO_3$	600	$CuSO_4.5H_2O$	0.6

Ratio—Ca: P = 1.88: 1.00

In a synthetic ration containing 5 per cent of salts I and 18 per cent casein the ratio of Ca: P = 1.37: 1.00.

Crude casein was purified for use in diet 448 by alternate solution in dilute NH_4OH, precipitation with dilute HCl, and washing of the precipitate with water. Five hundred grams of crude casein were dissolved in a 10-gallon volume of distilled water by addition of 500 cc. of 5 per cent NH_4OH. The casein was precipitated by addition of 5 per cent HCl to the isoelectric point of the casein. The supernatant serum was removed by siphoning, and the precipitate was washed in a 10-gallon volume of distilled water. Two additional precipitations were carried out, and the finally washed casein filtered and dried at 50°C. before a fan. The purified casein was fed in diet 448 at the level of 18 per cent, and 71 per cent of dextrin completed the ration.

Of thirteen animals receiving ration 448, all exhibited acute symptoms of B_4 deficiency. Eleven died during the fourth week of the experiment, the remaining two showing a decline in weight during the sixth week. When a vitamin B_4 supplement was added to ration 448, the vitamin $B(B_1)$ which was fed by pipette became a limiting factor, causing decreased food consumption and consequent decreased B_4 intake. All animals receiving ration 448 plus B_4 supplement had slight but definite symptoms of paralysis.

It was necessary to use a less destructible and more constant source of vitamin B(B_1). It was found possible to use a dried brewer's yeast (no. 118) obtained locally, which by assay was known to furnish sufficient vitamin B(B_1) for chicks at a level of 0.5 per cent. This yeast was added to diet 449 at the level of 1 per cent, and to diet 450 (table 1) at the level of 2 per cent, the latter of which proved most satisfactory, as is indicated in table 2. Growth charts of animals receiving diet 450 and diet 450 with added B_4 are presented in figure 1.

The gizzard factor. Routine post-mortem examination of all animals receiving basal diets 448, 449 and 450 consistently revealed the occurrence of lesions in the lining of the gizzard similar to those described by Dam and Schönheyder ('34). According to a system of grading the severity of these lesions, we used the terms normal, slight, marked, severe and very severe. The lesions were found in gizzards of all animals on the above-mentioned diets, and were graded either marked or severe (table 2). From previous experiments we had found that vacuum dried lung tissue has a preventative effect on the gizzard lesions, and levels as low as 5 per cent may be used to ensure a normal lining in the gizzard. Lack of the substance preventing occurrence of lesions in the gizzard has a profound effect upon the growth of these birds. Whether it is a primary or a secondary effect due to lack of absorption of other required factors has not been determined. Further evidence for the essential nature of this substance will be presented in a later paper. It was found necessary, following purification of the casein, to add a source of the factor preventing gizzard lesions.

In synthetic diet 451 this factor was supplied in 5 per cent vacuum desiccated lung tissue. This material replaced an equivalent amount of dextrin. In six of twelve birds receiving this ration the acute type of paralysis was observed during the third week of the experimental period followed by death within 48 hours, while the remaining six exhibited only slight symptoms, and continued their growth throughout the 6-week

period. Gizzard examination revealed only slight erosion areas in the gizzard lining, while with added B_4 (table 2) growth and gizzard lining were normal, and there was complete freedom from paralysis.

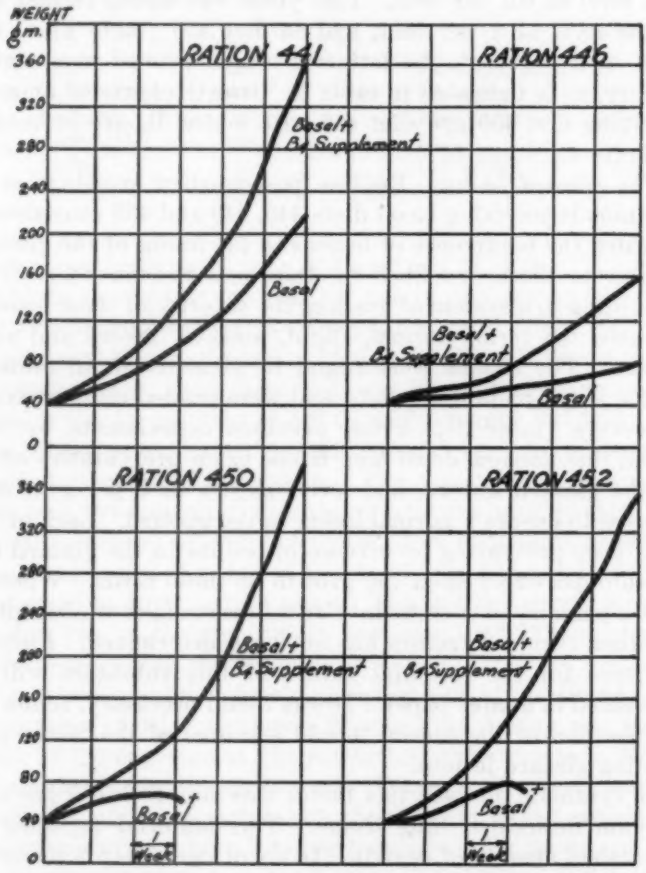

Fig. 1 Representative growth charts for the synthetic diets indicated, demonstrating the development of a successful synthetic diet for vitamin B_4 study in chicks.

A parallel study on the solubility of the factor preventing gizzard erosion showed it to be insoluble in water, giving a method for purifying the lung tissue of its probable vitamin B_4 content. This water extraction of the lung tissue was carried out, with a removal of 25 per cent solids, by extracting the finely ground lung substance three times with hot water. This material, added at the 5 per cent level in ration 452, gave satisfactory results with respect to normal gizzard lining and 100 per cent occurrence of acute vitamin B_4 deficiency. Addition of B_4 to this ration gave normal growth,

TABLE 3

Use of B_4 supplements in ration 452

SUPPLEMENT	NUMBER OF CHICKS	AVERAGE WEIGHT 3 WEEKS, GRAMS	AVERAGE WEIGHT 6 WEEKS, GRAMS	ONSET OF DEFICIENCY, WEEKS	NUMBER WITH DEFICIENCY	DEATHS PRIOR TO 6 WEEKS	SEVERITY OF GIZZARD LESIONS
10 per cent hog kidney	3	107	248	6	2 (slight)	0	Normal
15 per cent hog kidney	3	130	340	—	0	0	Normal
15 per cent hog brain	3	147	365	—	0	0	Slight
5 per cent peanut	6	83	—	3	6	6	Slight
10 per cent peanut	6	108	271	4	6 (slight)	0	Slight
15 per cent peanut	12	143	360	—	0	0	Normal
No supplement	26	78	—	3	26	26	Slight

normal gizzard lining, and complete freedom from paralysis. Constitution of ration 452 is given in table 1. The data for growth and occurrence of paralysis are found in table 2. In figure 1 are presented growth charts for animals receiving ration 452, and 452 with added vitamin B_4 supplement.

An illustration of the use of ration 452 in testing the vitamin B_4 potency of some food materials is given in table 3. Animal tissues were obtained fresh and carefully dried at 50°C. The peanuts used were unroasted, and kept in the shell until needed. They were dried at 50°C. before being ground with the synthetic ration. The rations were compounded

weekly to insure presence of sufficient vitamins A and B_4, factors which are inactivated by oxidation. It is readily seen that 15 per cent of dried brain tissue, dried kidney, or peanuts supplies sufficient vitamin B_4. These supplements at levels of 10 per cent or below were found to be inadequate as a source of vitamin B_4.

DISCUSSION

Our experience with the use of experimental synthetic diets for chicks has brought to our attention a number of interesting facts. Other workers have experienced similar phenomena, and some have been described in the literature, but a re-emphasis is necessary. In the first place, it is impossible to state that one has used a specific ration for the production of a certain nutritional deficiency. All we can say is that a ration has been compounded according to a definite formula. Where the ingredients used in a single laboratory vary from one experiment to another and with each new purchase, it is not surprising that even greater variations occur when the so-called similar rations are compounded in different laboratories. Perhaps one of the greatest offenders in synthetic rations used for both chicks and rats is casein. Casein manufactured at different times of the year certainly must vary when the nutritive value of the milk from which it is made varies. Extraction of casein with various solvents is probably not the most effective means of purifying casein. The particles are still intact and contaminants are not exposed to the solvent. We found that the greatest improvement in our ration depended upon the reprecipitation of the casein. Similar results have been obtained in studies with other vitamins and will be reported later.

Heat has not been found a satisfactory means of purification or separation of factors which do not differ greatly in their heat stability. There occurs an over-lapping destruction which does not give clearly defined results.

The reduction of the yeast in our diet was also a decided advantage. Although yeast is quite low in vitamin B_4, different batches undoubtedly vary in the amount of this factor

which they carry. The introduction of liver extract in place of part of the yeast seems to have a definite beneficial effect, although it is difficult at present to know if the liver extract supplies other essential factors, or if the known factors are more available.

Another fact which is readily evident is that we must give consideration to factors in the B complex other than B_1, B_2, B_4 and flavins. Upon greater refinement of our synthetic diet it has become necessary to add a supplement to prevent the gizzard lesions. Unless this factor is supplied it is impossible to make intelligent studies of vitamin B_4. Paralysis due to poor absorption may occur in chicks receiving ample amounts of B_4 if the chick is suffering from gizzard lesions. This is but another example of the interrelationship of the vitamins.

It is also important to mention that although we speak of ration 452 as one for the production of vitamin B_4 deficiency, it is impossible to conclude that it is complete in all other factors. We know that the addition of 15 per cent peanuts, brain, or kidney as sources of B_4 will produce normal chicks. We have also had fair success with certain B_4 concentrates, but until normal chicks are reared on ration 452 plus pure vitamin B_4 the possibility of still other factors must be considered. We also want to emphasize that ration 452 may not be a perfect ration for B_4 assay. Many improvements may be made in the future, but it is the best available at present.

The variation in the store of factors under question at the time an animal is placed on experiment will probably receive more and more attention. Studies on vitamins $B(B_1)$ (unpublished data) and D (Kline, Elvehjem and Halpin, '35) have demonstrated that feeding varying amounts of these factors to hens has no appreciable effect upon the rate at which chicks produced from these hens develop the respective deficiency diseases when placed on diets very deficient in these factors. It is evident from our work, however, that the vitamin B_4 storage of chicks from hens fed supplements high in this vitamin may be considerably increased. Somewhat similar results have been obtained in the case of the rat.

SUMMARY

1. A revised method for the study of vitamin B_4 in chicks has been described.

2. Changes in a synthetic ration for these studies, previously described, include purification of the casein and use of highly potent sources of the factors in the B complex other than B_4.

3. It was found necessary to add to the ration a substance which prevents the occurrence of lesions in the lining of the gizzard of the experimental chicks. Dried lung tissue is a potent source of this 'gizzard factor.'

4. On the improved basal synthetic ration a nutritional paralysis occurs in 100 per cent of the animals, which may be prevented by addition of supplements such as peanuts, brain, kidney or liver tissue.

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PELLAGRA-LIKE SYNDROME IN CHICKS

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ONE FIGURE

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As early as 1931, Ringrose and co-workers ('31) described the occurrence of a pellagra-like syndrome in chicks and later Kline et al. ('32–'33) reported "a method for the production of definite symptoms of pellagra in the chick" (Elvehjem and Koehn, '35). This method was successfully used in vitamin G (B_2) studies by Elvehjem and Koehn ('35) as well as by Stare ('35). Lepkovsky and Jukes ('35), however, conclude that the syndrome produced by the method of Kline is not due to a deficiency in "vitamin G, the pellagra-preventive (P-P) factor of Goldberger and co-workers ('28)." In view of these publications it seems desirable to report the following data obtained at these laboratories with chicks, wherein the observations are considered in parallel with studies of the dermatitis in rats (Bender et al., '36 and Ansbacher et al., '36).

EXPERIMENTAL

The chicks used were day-old single comb white Leghorns weighing from 35 to 45 gm. The experimental groups contained from fifteen to twenty individuals housed in wire screen compartments 24 × 26 × 18 inches, electrically heated and equipped with raised wire screen (3 mesh to the inch) bottoms. The basal rations were fed ad libitum and the various supplements were administered daily with a pipette.

Weights and observations of pathological symptoms were recorded twice a week. The data reported hereinafter were taken from comparable studies carried out over a period of about 7 months and involving approximately 800 chicks.

The basal ration no. 240-H proposed by Kline et al. ('32–'33) was employed. This ration consists of a mixture containing commercial casein, 12 parts; wheat middlings, 25 parts; yellow corn, 58 parts; and salt (common), 1 part; this mixture is heated to 95–100°C. for 144 hours and then cod liver oil, 2 parts, and calcium carbonate, 2 parts are added. Inasmuch as numerous data have accrued at these laboratories showing the variable character of the results obtained from vitamin studies wherein caseins of unknown history and different origin have been used, Kline's ration was modified for many of the comparative studies reported in the following. This modification consisted of heating the grain mixture in the absence of the commercial casein and substituting vitamin-free casein (Labco)[1] for the commercial casein. The unheated vitamin-free casein was mixed with the other components of the ration after such components as the grains and salt had been heated.

The results obtained from the unsupplemented basal ration containing the heated commercial casein and those obtained from the unsupplemented ration containing the unheated vitamin-free casein are shown in figure 1, graph 1. At the end of 5 weeks, 88 per cent of the chicks receiving the former ration showed the typical pellagra-like syndrome observed by various authors previously cited; 93 per cent of those receiving the unheated vitamin-free casein also showed the typical syndrome in severe form. These data would seem to indicate quite conclusively that the unheated vitamin-free casein (Labco) is fully as effective in permitting the development of the syndrome as the commercial casein heated to about 100°C. for 6 days. It is to be noted, however, that the rate of growth of the chicks receiving the heated commercial

[1] The Labco vitamin-free casein is distributed by The Casein Company of America, Inc., New York.

casein was somewhat greater than that of those receiving the unheated vitamin-free casein, notwithstanding the fact that the incidence and severity of the pellagra-like syndrome was substantially the same for both groups.

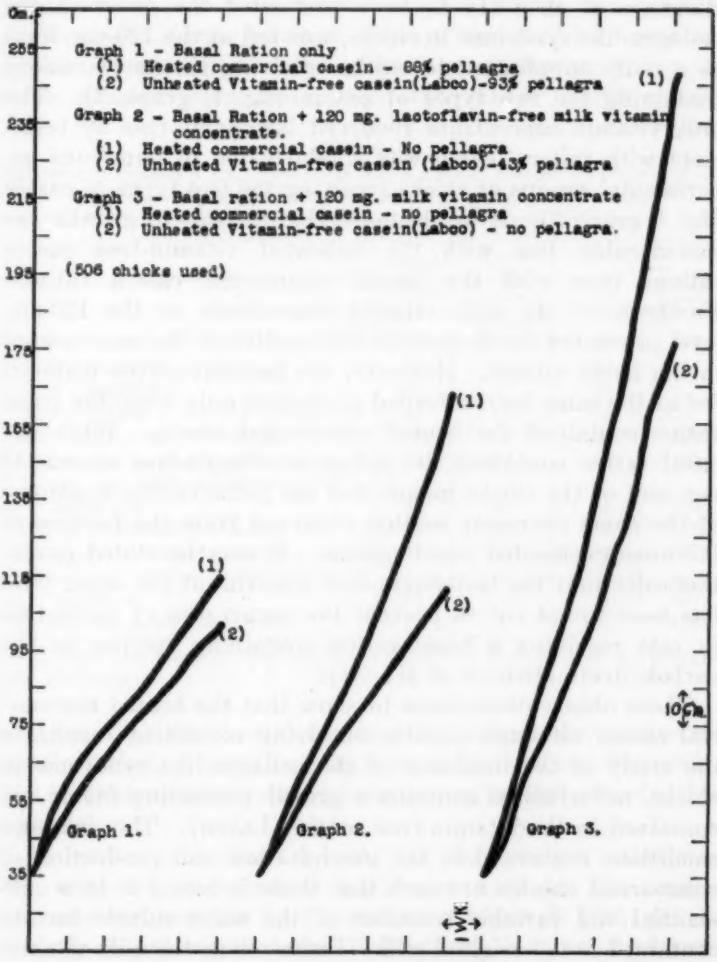

Fig. 1 The influence of commercial and vitamin-free casein on the rate of growth and incidence of the pellagra-like syndrome in chicks.

The significance of the difference in vitamin character of the two caseins is further illustrated by the results obtained from the comparative studies summarized in graphs 2 and 3 of figure 1. The milk vitamin concentrate, reported by Ringrose et al. ('31) to have prevented the onset of the pellagra-like syndrome in chicks, was fed at the 120-mg. level as a daily supplement to chicks receiving the basal rations containing the two types of casein (fig. 1, graph 3). The milk vitamin concentrate rendered lactoflavin-free by treatment with fullers' earth was similarly fed to numerous experimental groups of chicks receiving the two types of casein (fig. 1, graph 2). It will be noted that the rate of growth was considerably less with the unheated vitamin-free casein rations than with the heated commercial casein rations. Furthermore the milk vitamin concentrate at the 120-mg. level prevented the dermatitis irrespective of the caseins used in the basal rations. However, the lactoflavin-free material fed at the same level afforded protection only when the basal ration contained the heated commercial casein. When the basal ration contained the unheated vitamin-free casein, 43 per cent of the chicks manifested the pellagra-like syndrome of the same character as that observed from the feeding of the unsupplemented basal rations. It may be stated parenthetically that the lactoflavin-free material at the same level has been found not to prevent the occurrence of dermatitis in rats receiving a basal ration containing sucrose as the carbohydrate (Bender et al., '36).

These observations seem to show that the heated commercial casein although capable of giving consistent results in the study of the incidence of the pellagra-like syndrome in chicks, nevertheless contains a growth-promoting factor not contained in the vitamin-free casein (Labco). The unknown conditions concerned in the precipitation and production of commercial caseins are such that there is bound to be a substantial and variable retention of the water-soluble factors contained in the original milk. Inasmuch as the milk vitamin concentrate used in these studies gives results compatible in

respect to growth promotion with those obtained from commercial caseins, it is proper to assume that commercial caseins carry a substance or substances of the same character as those contained in the milk vitamin concentrate. The data indicate that such a factor or factors are heat-stable and growth-promoting. Furthermore it appears that fullers' earth removes at least a part of the antipellagra factor carried by the milk vitamin concentrate, since the fullers' earth treated

TABLE 1

Influence of vitamin B (B_1), lactoflavin and rice polish concentrate on growth and incidence of pellagra-like syndrome in chicks fed a basal ration containing unheated vitamin-free casein

SUPPLEMENTS	NUMBER OF CHICKS	PELLAGRA-LIKE SYNDROME INCIDENCE	AVERAGE WEIGHT AFTER	
			3½ weeks	5 weeks
		per cent		
None	83	93	87	102
12.5 γ vitamin B	16	88	97	...
10.0 γ lactoflavin	31	87	94	...
12.5 γ vitamin B and 10.0 γ lactoflavin	16	94	93	...
5 mg. rice polish concentrate	16	75	81	102
25 mg. rice polish concentrate	17	70	88	100
25 mg. rice polish concentrate + 7.0 γ lactoflavin	16	69	92	109
50 mg. rice polish concentrate	17	53	103	123
120 mg. rice polish concentrate	17	0	107	137
120 mg. rice polish concentrate + 7.0 γ lactoflavin	18	0[1]	115	150
240 mg. rice polish concentrate	17	0	97	135

[1] A small percentage of these chicks showed a very mild form of the pellagra-like syndrome.

concentrate, when supplementing the unheated vitamin-free casein ration, protected chicks only if fed at more than twice the level at which the concentrate not so treated afforded full protection.

In view of the above results the basal ration containing the unheated vitamin-free casein was used for the studies summarized in table 1. Crystalline vitamin B (B_1), fed as a daily supplement at the 12.5 γ level, had no significant influence on the incidence of the pellagra-like syndrome,

neither had 10.0 γ of crystalline lactoflavin nor the combination of these two crystalline products. However, the rice polish concentrate, previously shown to protect and cure rats from dermatitis (Bender et al., '36 and Ansbacher et al., '36), was found to contain the factor or group of factors which prevents the onset of the syndrome in chicks. At the 50-mg. level this concentrate protected about one-half of the experimental chicks; when fed in amounts of at least 120 mg., it afforded full protection. The influence of lactoflavin on the rate of growth is indicated in the experiments in which as little as 7.0 γ of crystalline lactoflavin was fed in conjunction with either 25 mg. or 120 mg. of the rice polish concentrate.

The results obtained from the use of the unheated vitamin-free casein (Labco) in place of the heated commercial casein in Kline's ration, seem to be of particular significance in directing attention to the basic importance of the character of the caseins used in studies concerning the various entities of the vitamin B-complex. If it were possible at the present time to determine with surety the exact biological value of the various caseins which have been employed for the various investigations reported heretofore, it is quite probable that many of the apparent discrepancies could be readily reconciled. The results reported herein seem to confirm the early observations of Goldberger and associates ('28) that the P-P factor can be adsorbed on fullers' earth. Lepkovsky and Jukes ('35) consider the possibility of a multiplicity of factors having antipellagric properties, because they and other investigators (Elvehjem and Koehn, '35; Stare, '35) were unable to confirm Goldberger's work. In reality these apparent discrepancies might conceivably be due in part at least to the character of the basal rations used particularly in respect to their casein constituent.

SUMMARY

The use of an unheated vitamin-free casein yielded consistent evidence in showing that the heated commercial casein as a constituent of the 240-H ration of Kline et al. ('32–'33) contains a factor or factors which accelerate the rate of growth and diminish the incidence of the pellagra-like syndrome in chicks.

Concentrates prepared from milk and from rice polish contain a factor or group of factors which prevents the onset of the pellagra-like syndrome in chicks.

Crystalline preparations of vitamin B and of lactoflavin do not prevent the development of the syndrome in chicks.

The factor or group of factors preventing the pellagra-like syndrome in chicks may be adsorbed on fullers' earth.

The data as a whole indicate that the factor or group of factors which prevents and cures the dermatitis in rats (Bender et al., '36 and Ansbacher et al., '36) is identical with that which prevents the pellagra-like syndrome in chicks.

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A STUDY OF THE SEASONAL VARIATION OF VITAMIN D IN NORMAL COW'S MILK[1]

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THREE FIGURES

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In recent years a great deal of attention has been given to the importance of vitamin D in the human dietary and particularly to the desirability and means of enhancing the milk supply in this factor. The methods by which the latter has been achieved are well known and relatively little thought is now given to the antirachitic potency of milk produced under ordinary farming conditions. Although many studies have been made of the vitamin D content of cow's milk, none of them has been of a comprehensive nature. Table 1 summarizes the results of some of these investigations.

Similar trends in variations of the vitamin D potency of milk are indicated by the work of other investigators. Luce ('24) found milk from pastured cows definitely richer in vitamin D than that from stall-fed cows kept in the dark. Luce concluded that the concentration of the antirachitic factor in milk depended on the ration and possibly also on the degree of illumination received by the cow. Chick and Roscoe ('26) found 0.25 gm. of milk fat from pastured cows equal to 0.60 gm. milk fat from cows fed green feed in a dark stall. Exposure of the cow to outdoor light without a change in ration resulted in a twofold increase in the vitamin D content of

[1] The data in this paper were taken from the thesis presented by H. Ernest Bechtel to the faculty of the Graduate School of Michigan State College in 1935 in partial fulfillment of the requirements for the degree of doctor of philosophy.

the milk fat. Dutcher and Honeywell ('27) examined some Kansas butter samples and found that milk fat from cows exposed to sunshine was superior in vitamin D potency to fat produced by cows fed in the dark.

These and other studies which might be mentioned indicate in a general way the variations that occur in the vitamin D potency of milk and the factors which contribute to this variability. However, most of the work done has been of a rather fragmentary nature so that it seemed desirable to make a more extended study of the subject.

TABLE 1

SOURCE OF DATA	VITAMIN D CONTENT OF NORMAL MILK UNITS PER QUART		
		U.S.P.[1]	Steenbock
Steenbock and associates (Wisconsin) ('30)	June 1925	21.3	7.9
	September 1925	36.5	13.5
Mitchell and associates (Pennsylvania) ('32)	Summer milk	16.2	6.0
Krauss (Ohio) ('33)	Winter milk	7.6	2.8
	Summer milk	16.2	6.0
Russell (New Jersey) ('33)	Summer milk		
	Always less than	43.2	16.0

[1] The values in this column were obtained by multiplying the number of Steenbock units by 2.7.

MATERIALS AND METHODS

This study was begun in 1932, the milk being derived from various sources. Milk from the Holstein and Guernsey herds at Michigan State College was assayed monthly over a period of 2 years. Only the higher producers, namely, cows that were being milked three times daily were used, the average number of Holstein and Guernsey cows being fourteen and eight, respectively, for the 2-year period. From these cows 100-pound portions of composite 24-hour samples were saved monthly.

Monthly samples of a similar nature were derived from a few of the highest producing cows in the Holstein herd of the Michigan Experiment Station. These cows were kept on a ration of alfalfa hay, silage and corn.

From July 1933 to July 1934 monthly samples of Michigan State College Creamery butter, made from milk produced by local Michigan dairymen, were also put aside for vitamin D assays. Approximately fifteen herds, consisting largely of grade Holstein cows were represented in this group of samples.

In the case of the milk a small portion was used for a fat determination, the remainder being run through a cream separator. The butter obtained by churning the cream was packed in paper cartons and stored at 0°F. until the samples were to be assayed. At this time the butter was heated on a steam bath for about an hour and the relatively pure milk fat upon which the assays were conducted was suctioned off. Except when needed for assay, the fats were kept at 0°F.

The vitamin D determinations were carried out by the curative feeding technic, several changes being made in the official procedure. The Steenbock basal ration was slightly modified to obtain more consistent and somewhat better growth of the rats during the preliminary period. The rachitogenic diet used throughout this study was composed of the following:

	Per cent
Yellow cornmeal	38.0
Oatmeal	37.5
Wheat gluten	20.0
Calcium carbonate	3.0
Sodium chloride	1.0
Yeast powder	0.5

Instead of feeding the fat as a daily supplement during the first 8 days of the test period, the entire amount was mixed with 40 gm. of the basal diet. This mixture was found to be consumed in 6 to 8 days after which the basal diet was fed to finish the 10-day period. Control rats receiving 29 mg. of Official Reference Oil equivalent to 2.7 U.S.P. units were used for comparison. The usual staining technic was applied to the radii and ulnae.

In carrying out the assays a preliminary test was made to determine the approximate vitamin D content of the vari-

ous samples. The confirmatory tests were then conducted at three levels using three to five rats at each level.

It became apparent early in this investigation that fats of low potency could not be assayed because of the limited capacity of the rats to consume fat. Amounts up to 6 and 8 gm. were consumed fairly consistently. However, when the dosage was increased to 10 gm. approximately only half of the test animals consumed the fat-basal diet mixture in 8 days. Attempts were therefore made to effect a concentration of the vitamin D so that fats of lower potency might be assayed. Although the work of Kon and Booth ('34) indicated that at least a part of the vitamin D of butter fat was unstable and could not be recovered quantitatively in the non-saponifiable matter, their method as well as several modifications were given a trial. Sometimes the recovery of vitamin D was quantitative, but more often it was not, so that this method of concentration was abandoned.

Inasmuch as the concentration of vitamin D in cod liver oil may be accomplished by extraction of the oil with alcohol, this method was next tried and proved to be satisfactory. The fats of low potency were therefore treated in the following manner: 100 gm. of melted milk fat was placed in a separatory funnel previously warmed in a 37°C. oven and 100 cc. of hot ethyl alcohol (95 per cent) was added. The mixture was then shaken fairly vigorously and the funnel placed into the 37°C. oven until the layers had separated. The fat was then drawn off into a warm beaker and the alcohol layer into a 500 cc. volumetric flask. The fat was returned to the separatory funnel and the beaker rinsed with 50 cc. of hot alcohol which was then added to the fat. The mixture was again shaken and the layers allowed to separate in the oven. The separations were made as before and three additional extractions carried out with 50 cc. portions of hot alcohol. By this process approximately 20 per cent of the fat was removed and this fat contained all of the vitamin D. The combined extracts were brought to a volume of 500 cc. with ether in order that the fat would be kept in solution. It was observed

that the antirachitic value was retained for at least 2 months when the extract was stored at 0°C. Aliquots of this solution were poured on 40 gm. portions of the rachitogenic diet in evaporating dishes and the ether and alcohol allowed to evaporate spontaneously. These mixtures were then fed in the usual manner.

TABLE 2

Antirachitic potency of milk fat obtained from the Holstein herd[1]

DATE	FAT-CONTAINING 1 U.S.P. VITAMIN D UNIT	AVERAGE DAILY PRODUCTION PER COW		FAT IN MILK	VITAMIN D PER QUART OF MILK		AVERAGE DAILY AMOUNT OF SUNSHINE
		Fat	Vitamin D		U.S.P.	St.[2]	
	gm.	*gm.*	*U.S.P.*	*per cent*			*hours* [3]
1932							
July	1.5	817	545	3.26	17.8	6.6	11.5
August	1.1	717	701	2.88	20.2	7.5	10.0
September	1.1	862	784	3.53	27.7	10.3	7.8
October	3.3	748	227	2.78	6.3	2.3	3.3
November	—	—	—	—	—	—	4.0
December	—	—	—	—	—	—	3.0
1933							
January	5.6	826	148	3.15	4.6	1.7	4.1
February	9.3	875	94	3.26	3.1	1.1	7.1
March	3.7	835	226	2.97	6.7	2.5	4.4
April	3.7	748	202	2.93	5.9	2.2	6.8
May	3.0	730	243	2.89	7.0	2.6	8.5
June	—	—	—	—	—	—	12.7
July	1.5	776	517	3.20	16.6	6.1	12.8
August	1.9	862	454	3.28	14.9	5.5	10.8
September	1.5	780	520	2.98	15.5	5.7	7.1
October	3.7	690	186	2.96	5.5	2.0	6.0
November	7.4	753	102	3.19	3.2	1.2	2.0
December	—	—	—	—	—	—	1.4
1934							
January	—	—	—	—	—	—	2.3
February	7.4	844	114	3.17	3.6	1.3	5.9
March	7.4	939	127	3.29	4.2	1.6	5.0
April	4.4	871	198	3.06	6.1	2.3	6.0
May	3.0	898	299	3.31	9.9	3.7	11.3

[1] Average of fourteen cows per month.

[2] Steenbock.

[3] Average amount of available sunshine according to the East Lansing Weather Bureau.

The above method permitted practically complete recovery of the vitamin D from samples of milk fat of which 2 to 10 gm. had to be fed of the original fat to get the typical narrow continuous line of calcification. Inasmuch as there was no way of checking the fats of lower potency directly, it had to be assumed that the method was also satisfactory for such

TABLE 3

Antirachitic potency of milk fat obtained from the Guernsey herd[1]

DATE	FAT CONTAINING 1 U.S.P. VITAMIN D UNIT	AVERAGE DAILY PRODUCTION PER COW		FAT IN MILK	VITAMIN D PER QUART OF MILK		AVERAGE DAILY AMOUNT OF SUNSHINE
		Fat	Vitamin D		U.S.P.	St.[2]	
	gm.	*gm.*	*U.S.P.*	*per cent*			*hours* [3]
1932							
July	1.1	649	590	4.93	43.8	16.2	11.5
August	1.1	685	623	4.86	43.1	16.0	10.0
September	1.3	708	545	5.15	38.7	14.3	7.8
October	3.0	699	233	4.52	14.7	5.4	3.3
November	3.7	708	191	4.02	10.6	3.9	4.0
December	3.7	930	251	4.76	12.6	4.7	3.0
1933							
January	4.4	907	206	4.42	9.8	3.6	4.1
February	9.3	835	90	4.57	4.8	1.8	7.1
March	3.7	762	206	4.33	11.4	4.2	4.4
April	3.0	658	219	4.34	14.1	5.2	6.8
May	3.0	694	231	4.42	14.4	5.3	8.5
June	—	—	—	—	—	—	12.7
July	1.5	744	496	4.43	28.8	10.7	12.8
August	1.9	853	449	5.09	26.2	9.7	10.8
September	1.9	694	365	4.61	23.7	8.8	7.1
October	2.6	721	277	4.76	17.9	6.6	6.0
November	3.7	912	246	4.86	12.8	4.7	2.0
December	—	—	—	—	—	—	1.4
1934							
January	—	—	—	—	—	—	2.3
February	7.4	866	117	4.44	5.9	2.2	5.9
March	5.6	835	149	4.50	7.8	2.9	5.0
April	3.7	826	223	4.52	11.9	4.4	6.0
May	3.0	871	290	4.90	15.9	5.9	11.3

[1] Average of eight cows per month.

[2] Steenbock.

[3] Average amount of available sunshine according to the East Lansing Weather Bureau.

TABLE 4

Antirachitic potency of milk fat obtained from the experiment station herd[1]

DATE	AVERAGE DAILY FEED INTAKE			FAT CONTAINING 1 U.S.P. VITAMIN D UNIT	AVERAGE DAILY PRODUCTION PER COW		FAT IN MILK	VITAMIN D PER QUART MILK		DAILY AMOUNT SUNSHINE
	Alfalfa	Silicon	Corn		Fat	Vitamin D		U.S.P.	St.[2]	
	lb.	*lb.*	*lb.*	*gm.*	*gm.*	*U.S.P.*	*per cent*			*hours*[3]
1933										
July	24.2	—	15.1	1.3	544	418	2.30	17.3	6.4	12.8
August	26.3	—	13.8	1.5	617	411	2.64	17.2	6.4	10.8
September	26.4	5.5	8.3	1.5	581	387	3.18	20.7	7.7	7.1
October	23.1	25.9	4.4	2.6	508	195	3.34	12.5	4.6	6.0
November	26.0	20.9	3.5	3.7	662	179	4.02	10.6	3.9	2.0
December	24.9	24.0	4.1	3.7	435	118	3.29	8.7	3.2	1.4
1934										
January	—	—	—	—	—	—	—	—	—	2.3
February	26.1	26.0	0.3	4.4	327	74	3.40	7.5	2.8	5.9
March	21.2	19.7	3.2	4.4	531	121	3.73	8.3	3.1	5.0
April	23.8	13.0	9.3	3.7	535	145	2.84	7.5	2.8	6.0
May	26.0	3.6	12.6	2.2	794	361	3.13	13.9	5.1	11.3

[1] Average of five cows per month.

[2] Steenbock.

[3] Average amount of available sunshine according to the East Lansing Weather Bureau.

TABLE 5

Antirachitic potency of creamery milk fat[1]

DATE	FAT CONTAINING 1 U.S.P. VITAMIN D UNIT	FAT IN MILK	VITAMIN D PER QUART OF MILK		AVERAGE DAILY AMOUNT OF SUNSHINE
			U.S.P.	Steenbock	
	gm.	*per cent*			*hours*[2]
1933					
July	1.3	3.5	26.3	9.7	12.8
August	1.9	3.5	18.0	6.7	10.8
September	1.3	3.5	26.3	9.7	7.1
October	2.6	3.5	13.1	4.9	6.0
November	4.4	3.5	7.8	2.9	2.0
December	3.0	3.5	11.4	4.2	1.4
1934					
January	—	—	—	—	2.3
February	3.7	3.5	9.2	3.4	5.9
March	—	—	—	—	5.0
April	—	—	—	—	6.0
May	3.0	3.5	11.4	4.2	11.3
June	1.9	3.5	18.0	6.7	11.8

[1] Average of twelve herds of cows per month.

[2] Average amount of available sunshine according to the East Lansing Weather Bureau.

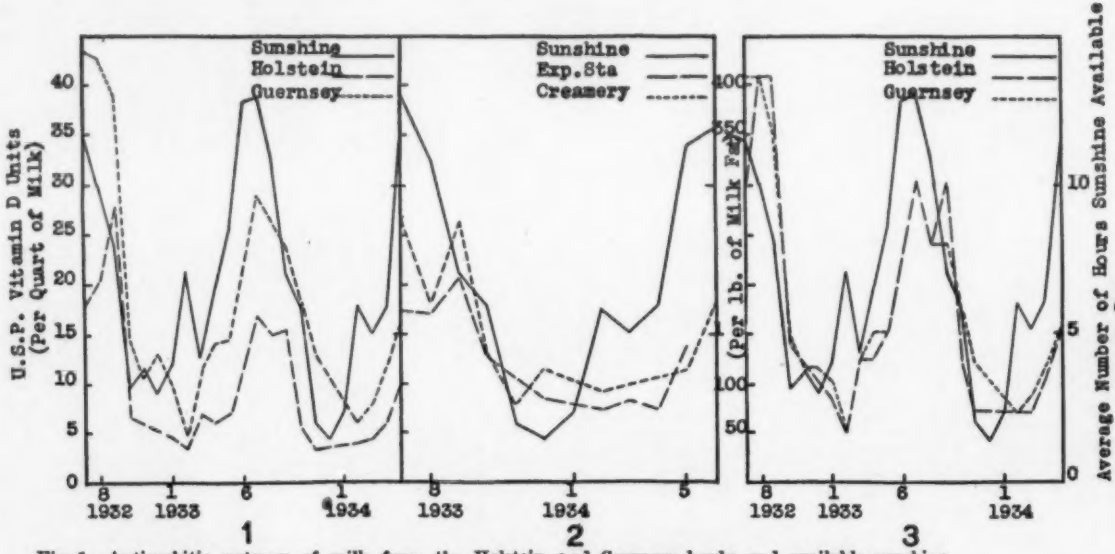

Fig. 1 Antirachitic potency of milk from the Holstein and Guernsey herds, and available sunshine.
Fig. 2 Antirachitic potency of milk from the Experiment Station herd and M. S. C. Creamery, and available sunshine.
Fig. 3 Antirachitic potency of milk fat from Holstein and Guernsey herds, and available sunshine.

fats. Quantitative recovery was also obtained when a definite amount of vitamin D from the official reference oil was added to milk fat and subjected to hot alcohol extraction.

The results of this study of the seasonal variation in the vitamin D content of cows' milk are presented in tables 2, 3, 4 and 5 and portions of the data are shown graphically in figures 1, 2 and 3. The data include not only the results of the bioassays but also the average daily production of milk fat and the number of vitamin D units in the milk fat. The results were also calculated in terms of U.S.P. units per quart and these values are presented in figures 1 and 2 with the average daily hours of sunshine available each month. To simplify the comparison of these results with those given in the older literature the antirachitic potency is also expressed in terms of Steenbock units.

Inasmuch as exposure of the cows to sunlight and the ingestion of sun cured roughages are two important factors which influence the vitamin D potency of the milk, a brief reference to the general management of the several dairy herds is appropriate. The main Holstein and Guernsey herds of the college were kept under parallel conditions at all times. From May to September, inclusive, these animals were pastured an average of 8 hours daily and received no hay or corn silage. During October they were pastured an average of 5 hours daily and received about 1 pound of hay per 100 pounds of body weight. From November to April, inclusive, the animals were put out doors in dry lot for an average of 2 hours daily. During this period in 1932–1933 they received besides their allowance of grain an average of approximately 2 pounds of hay per 100 pounds of body weight, no corn silage being included. During the corresponding period in 1933–1934 the animals received ¾ pound of hay and 3 pounds of corn silage per 100 pounds of body weight in addition to grain. The average weight of the Guernsey cows was 1150 and that of the Holsteins 1400 pounds.

The Holstein cows in the experiment station herd were out of doors in dry lot an average of 7 hours daily from May

to September, inclusive, and about 2 hours daily during the other months. These cows were kept on a ration of alfalfa hay, corn silage and corn as shown in table 4.

The general management of the local Michigan dairy herds which served as the source of the college creamery butter samples was typical of that practiced in this state. The cows were fed chiefly home grown feeds consisting largely of alfalfa and cereal grains and were usually pastured as early and as late as conditions permitted. They were probably exposed to sunshine for a longer time than the cows in the college herds.

Regarding the assaying of the various samples of butter, there was a considerable interval between the time the samples were obtained and the time the bioassays were made. This delay was due chiefly to the fact that a satisfactory method had to be developed before the samples of low vitamin D potency could be assayed. However, there appeared to be no danger of a loss of antirachitic potency because some of the older samples were assayed 30 months after the first test was completed, the results indicating that vitamin D in milk fat is stable for at least 30 months when the samples are stored at 0°F. in the dark.

Practically all of the results given in tables 2, 3, 4 and 5 were obtained by using the alcohol extraction method, although most of the summer samples and a few of the more potent winter samples were also assayed by feeding the original fat.

DISCUSSION

In this investigation two assumptions were made which appear to be justifiable. It was assumed that all of the antirachitic potency of cows' milk is present in the milk fat and that there was no significant loss in potency incidental to the separating and churning of the cream.

The standard curative feeding technic was selected for the bioassays because this method has a number of definite advantages over the prophylactic procedure. Besides the fact that the former is much more widely used, it permits the

feeding of relatively large amounts of fat without interfering with the test itself. In the prophylactic method the addition of vitamin D free fat to the basal rachitogenic diet will of itself cause a definite increase in the ash content of the bones, the increase depending on the amount of fat added. In this connection the slight modification of the rachitogenic diet seems justified because the rats attained a slightly larger size at the end of the preliminary period and had somewhat better appetites. This permitted the feeding of larger amounts of fat which was necessary in the case of the samples of lower potency. Nevertheless there were limitations in the capacity of the rachitic rats to consume fat and this necessitated concentration of the vitamin D. The alcohol extraction method described above seemed to solve this difficulty.

Inasmuch as Kon and Booth ('34) had felt that the vitamin D in winter milk might be different from that in summer milk because of the difference in stability to saponification, some of the more potent winter samples were assayed both by feeding the original fat and an equivalent amount of the extract. Although many of the rats failed to consume the larger doses of fat during the first 8 days of the experimental period, a sufficiently large number was used so that an assay at the 10-gm. level was made possible. The results indicated that there was no apparent loss in vitamin D in making the alcohol extractions of the winter samples tested, and served as the basis for the assumption that the assays of fats of still lower vitamin D potency by this method of concentration might be reliable.

The results obtained demonstrate that milk produced by cows managed under practical farming conditions varied as much as 900 per cent in antirachitic potency, reaching a maximum from June to September and beginning with October, declining rapidly to a minimum which usually occurred in February. From the assays made on the milk fats it was calculated that the maximal potency of the milks examined in this study was 43.8 U.S.P. units per quart. Values of 20 to 30 units per quart were not uncommon during the summer

months whereas values of 8 units and less were frequently observed during the winter months. These results in a general way corroborate those of other investigators.

Regarding the factors which contribute to the variability in the vitamin D content of milk, the amount of exposure of the cows to sunlight probably plays the major role. This is strikingly indicated by the excellent correlation between the vitamin D potency of the milk and the amount of available sunshine as shown in figures 1, 2 and 3. Undoubtedly even better correlation might have been obtained if a record had been kept of the hours of actual exposure to sunlight as well as of the ultraviolet intensity of the sunlight. The lack of agreement during February is to be explained on this basis.

It follows from the above that the vitamin D contained in ordinary dairy feeds, particularly roughages and silage, however important this source may be to the general well being and productiveness of the dairy cow, contributes relatively little to the vitamin D content of the milk. Furthermore the rapid drop in the antirachitic potency of milk which follows the decrease in exposure of the cows to sunlight suggests that under ordinary conditions of management and feeding the dairy cow has practically no opportunity to build up a reserve of vitamin D during lactation.

In comparing the Holstein and Guernsey samples as shown in tables 2 and 3 it is interesting to note that there was little difference in the antirachitic potency of the milk fat. However, because of the higher per cent of fat in the milk of the latter breed, the calculated vitamin D content of the milk was greater.

SUMMARY

1. A method is presented for the concentration of the antirachitic factors in milk fat thus making possible the biological assay of fats of low potency.

2. The monthly assay of milk fats from several sources over a period of 2 years shows that milk may vary as much as 900 per cent in antirachitic potency. Highest values were obtained during July, August or September and lowest usually in February. Vitamin D values ranging from 4.8 to 43.8 U.S.P. units per quart of milk were observed in the case of Guernsey milk whereas the extreme values for Holstein milk were 3.1 to 27.7 U.S.P. units per quart.

3. The close correlation between the antirachitic potency of milk and the amount of available sunshine indicates that the exposure of cows to sunlight is the major factor contributing to the vitamin D content of milk.

4. Apparently the cow has little or no opportunity to store vitamin D during lactation under ordinary dairy management conditions.

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THE RELATION BETWEEN CALCIUM RETENTION AND THE STORE OF CALCIUM IN THE BODY, WITH PARTICULAR REFERENCE TO THE DETERMINATION OF CALCIUM REQUIREMENTS [1]

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ONE FIGURE

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The requirement of protein for the growth of an animal is measured by the retention of nitrogen under conditions favoring the most rapid rate of growth. This is true because all (or practically all) of the nitrogen retained during growth becomes an integral part of the protoplasmic or structural framework of the newly developed tissues. Very little if any of the nitrogen so retained is laid down as an inert deposit subject to mobilization when the needs of the tissues for protein and other nitrogenous complexes are not adequately covered by the dietary supply. Under these conditions the nitrogen retained daily measures the day to day need for this element.

In the case of other indispensable nutritive elements, such as calcium, phosphorus and iron, the situation is quite different. With each of these elements there is provision in some of the tissues for an inert deposit subject to call, as the needs of the body dictate. In the case of calcium and phosphorus these inert deposits perform the purely mechanical

[1] This investigation was made possible by the donation of funds to the University of Illinois by the American Dry Milk Institute, Inc.

function of giving rigidity to the skeletal and cartilaginous framework of the body, but they are, nevertheless, a readily available source of supply for calcium (McCrudden, '12; Bauer, Aub and Albright, '29) and phosphorus to the soft tissues as the need arises.

With such elements the requirements for animal growth are not necessarily measured by their rate of retention in the body under conditions favoring the most rapid development of the tissues, for two reasons. In the first place, the retention may include a variable fraction used to fill up depleted stores. This fraction is not a part of the day to day requirement for the element in question, since it is the expression merely of a preceding state of inadequate nutrition with reference to it.

In the second place the retention may include a fraction used to fill to repletion the stores developing in the new tissues although repletion may not be required for maximum physiological efficiency. It is quite conceivable, with reference to calcium for example, that the skeleton of a rat would hold 65 per cent of ash, but that a content of 50 per cent is all that is needed to maintain a proper rigidity of the bones, and a normal content of calcium in the soft tissues in so far as this content is dependent upon the calcium content of the skeleton.

At the present time, the existence of the latter fraction is somewhat hypothetical though not improbable. Sherman and Booher ('31) have shown that the calcium content of rats growing on rations varying in their content of this element from 0.16 to 0.50 per cent is closely dependent upon the level of dietary calcium. Sherman and Campbell ('35) proved that a diet containing 0.2 per cent of calcium was deficient in this nutrient for rats as indicated by impairment of growth, vitality, fertility and ability to nourish the young. Unfortunately for the purposes of this discussion the carcasses and the bones of these rats were not analyzed for calcium. On the other hand it has been abundantly demonstrated (Friedman, '35) that the human infant retains the calcium from

cow's milk at a rate two to three times as rapid as that of breast milk in conformance with the much larger content of calcium in cow's milk (0.114 per cent) than in human milk (0.031 per cent). It is doubtful that any benefit is derived from this more rapid rate of bone calcification. Hence, there is room for the belief that benefits accrue to increasing rates of calcification of the skeleton only up to a point considerably short of complete saturation. If this is true for the growing animal, it may be equally true that in the adult maximum physiological efficiency is consistent with incomplete saturation of the bones with calcium. However, until the minimum percentage saturation of the calcium stores compatible with maximum physiological performance has been determined, it would seem to be the wiser course (certainly the safer course) to consider complete saturation of the stores as the ideal condition, and to include in the calcium requirement for growth (or maintenance) that amount needed to produce (or maintain) a condition of full repletion.

The highly variable retentions of calcium by children reported in the literature may conceivably be due to the variable condition of their skeletal tissues with respect to calcium saturation. This has been emphasized particularly by Daniels and her associates ('34) and by Wang, Kaucher and Frank ('28). A variable saturation of the calcium stores of the experimental subjects is probably an important factor in the discrepancies among estimates of the calcium requirements of children: For example, whether these daily requirements may be largely covered by the calcium contained in a pint of milk or less (Daniels, Hutton, Knott, Wright and Forman, '35), a quart of milk (Sherman and Hawley, '22), or more than a quart of milk (Jeans and Stearns, '32). In fact, Daniels and co-workers ('35) interpret high retentions of calcium in children as being due to previous depletion, and Clark ('26) in his studies on the calcium retentions of adults likewise correlates high retentions with previous inadequate nutrition. However, these interpretations are not based upon definite evidence of the pre-experimental state of the calcium stores

of the subjects, while, in apparent contradiction to them, Wang, Kaucher and Frank ('28) observed that their underweight subjects did not store calcium at a much more rapid rate than their vigorous normal subjects; also no correlation is evident in these experiments between the daily calcium retention per kilogram of body weight and the percentage under weight of the children.

Boldt, Brahm and Andresen ('29) have reported some experimental evidence for the belief that previous depletion of calcium stores will accelerate its storage. The calcium and phosphorus metabolism of two infants was studied over a period of 3 months, divided into three experimental periods. In the first and third periods, the infants were given human milk, and in the second period cow's milk containing a much greater content of Ca and P. The retentions of these elements were highest in the second period, but in the third period the retentions were much lower than in the first period, although the intake of calcium was somewhat greater. The results may be explained on the plausible assumption that the calcium stores were more nearly repleted at the start of the third period than at the start of the first, the greater degree of repletion inducing a lower rate of calcium deposition.

The problem of the effect of the previous condition of the calcium stores on the rate of retention of calcium during periods of experimental feeding is important to the determination of the calcium requirement of man and of all animals, and since available information appears insufficient to solve the problem, the experiment to be reported in this paper was carried out.

PLAN OF EXPERIMENT

In this experiment the calcium stores of rats were filled to different levels by subsistence in a preliminary period on diets varying in their content of calcium from 0.18 to 1.25 per cent. In a subsequent period all rats were placed upon the diet containing 1.25 per cent of calcium and their retentions of this element were then measured both by carcass analysis and by collection and analysis of urine and feces.

The paired-feeding method was used throughout these experiments in order that the comparative effects of the diets upon the calcium stores of the rats would relate solely to the calcium contents of the diets used, and not to the amounts of them consumed.

In the preliminary period three groups of rats, each consisting of eleven or twelve pairs, were used in three comparisons: First, a comparison of a ration containing 0.18 per cent of calcium with one containing 1.25 per cent; second, a comparison of a ration containing 0.32 per cent calcium with one containing 1.25 per cent; and third, a comparison of a ration containing 0.49 per cent calcium with one containing 1.25 per cent. The rats were started at weights of 40 to 60 gm. and were fed in this manner for 28 days in the case of the first two comparisons, but for only 14 days in the case of the last comparison. During this time the growth of the rats was somewhat restricted by restriction of food intake. At the termination of the preliminary period, three or four pairs of rats in each of the three series were sacrificed for chemical analysis. The results of this analysis afforded information of the calcium saturation of the carcasses effected by the various experimental diets. Compared with analyses of control rats analyzed at the initial experimental weights, estimates of the relative storages of calcium on the various diets are possible.

At the end of the preliminary period all rats were placed upon the highest level of calcium, 1.25 per cent, eight pairs in each of the three series being continued in this manner, still retaining the paired-feeding technic. On four pairs in each series, the combined excreta were collected daily and composited weekly for analysis of their calcium content. Feces markers of Fe_2O_3 or Cr_2O_3 were used at the end of each experimental week. When the rats reached approximately 200 gm. in weight, or somewhat more, they were all sacrificed for measurement and analysis. During this period, growth was not restricted and the intake of food was such that the desired final weights were reached in some pairs in only 2 to 3 weeks; in a few pairs, in 5 to 6 weeks.

The rations used all contained 23.21 per cent of dried fat-free whole egg, 10 per cent of sucrose, 10 per cent of lard, 3.43 per cent of a salt mixture[3] containing no calcium or phosphorus, 2 per cent of cod liver oil, 7 per cent of dried yeast, 39.36 per cent of cornstarch, and 5 per cent of a mixture of $BaSO_4$ and $CaHPO_4.2H_2O$, the components of which were adjusted to give the desired percentage of calcium.

The rations contained an average of 20.75 per cent of protein ($N \times 6.25$) and 4.47 calories per gram. The average contents of calcium and phosphorus were 0.185 and 0.354 per cent, respectively, for diet A, 0.318 and 0.484 per cent for diet B, 0.487 and 0.574 per cent for diet C, and 1.252 and 1.121 per cent for diet D. Calcium was determined in food, combined excreta, and carcasses by the method of McCrudden ('10); phosphorus was determined by the Pemberton-Kilgore method as modified by Hibbard ('13).

EXPERIMENTAL RESULTS

It is not necessary to give the individual body weight records of the rats during the preliminary period because they have no intimate bearing on the problem under study. In the comparison of the 0.18 and 1.25 per cent calcium levels, the rat on the higher level of calcium, but receiving the same amount of food as his pair mate, gained the faster in eight of the eleven pairs, the average excess gain being 2.08 gm., the standard deviation of differences in gain between pair mates being 3.87 gm., and the probability that this was a

[3] The mineral mixture contained:

	Per cent
Potassium citrate, $K_3C_6H_5O_7.H_2O$	20.52
Potassium sulfate, K_2SO_4	2.08
Potassium chloride, KCl	19.61
Ferric citrate, $FeC_6H_5O_7.1.5\ H_2O$	1.48
Potassium iodide, KI	0.0047
Manganese sulfate, $MnSO_4$	0.0184
Sodium fluoride, NaF	0.0578
Potassium aluminum sulfate, $K_2Al_2(SO_4)_4$	0.0057
Sodium chloride, NaCl	9.07
Magnesium chloride, $MgCl_2.6H_2O$	47.17

purely fortuitous outcome being 0.059 according to the method of analysis of Student ('08). This difference in gain is slight but on the borderline of statistical significance. In the other comparisons the differences in gain between pair mates were quite insignificant, the pairs dividing five to six in favor of the low calcium level in the comparison of the 0.32 and 1.25 per cent calcium rations, and five to seven in favor of the lower level in the comparison of the 0.49 and 1.25 per cent calcium rations. The daily gains in body weight in the three comparisons averaged 2.33, 2.81, and 5.07 gm., respectively.

The results of the analysis for calcium of the carcasses of the rats killed at the termination of the preliminary period are summarized in table 1. The average percentages of calcium (computed on the empty weight) in the first comparison were 0.698 for the rats on the ration containing 0.18 per cent of calcium and 0.895 for the rats on the ration containing 1.25 per cent of calcium. In the second comparison, the percentages were 0.704 for the rats on the ration containing 0.32 per cent of calcium and 0.820 for the rats on the ration with a calcium content of 1.25 per cent. In the last comparison the average percentages were 0.682 and 0.733, respectively, for the rations containing 0.49 and 1.25 per cent of calcium.

Although the numbers of paired analyses in each comparison were small, the differences between pair mates were quite definitely significant statistically in all cases, as the computations of table 2 indicate. The probabilities given in the last column of the table are so small that they may be neglected. Consequently it may be concluded rationally that the observed differences in the calcium content of paired rats were produced by the different levels of calcium in the diets upon which the rats had subsisted. In other words, the different levels of calcium in the experimental diets had in fact induced different degrees of saturation of the calcium stores. Hence, the purpose of the preliminary period of feeding was realized.

It will be noted that there was considerable variation among the calcium contents of rats reared upon the same experimental diets. This is particularly noticeable with the rats

TABLE 1

Calcium content of selected rats at termination of preliminary period

PAIR NUMBER	PER CENT CA IN RATION	BODY WEIGHT		AVERAGE DAILY GAIN IN BODY WEIGHT	PER CENT CA IN CARCASS [1]
		Alive	Empty		
		gm.	*gm.*	*gm.*	
1	0.18	123	118	2.3	0.66
	1.25	124	123	2.5	0.82
3	0.18	102	98	2.1	0.70
	1.25	105	103	2.4	0.98
11	0.18	100	97	1.8	0.73
	1.25	105	101	2.0	0.88
2	0.32	140	136	3.4	0.58
	1.25	139	137	3.2	0.70
5	0.32	116	114	2.5	0.74
	1.25	115	113	2.5	0.85
11	0.32	98	97	1.8	0.80
	1.25	108	106	2.1	0.91
1	0.49	103	101	4.3	0.67
	1.25	103	102	4.2	0.69
2	0.49	94	93	3.9	0.70
	1.25	97	95	3.8	0.72
3	0.49	138	137	6.0	0.69
	1.25	137	137	6.0	0.76
10	0.49	137	136	5.1	0.67
	1.25	139	138	5.3	0.76

[1] Computed on the empty weight.

TABLE 2

Statistical analysis of the calcium percentages summarized in table 1

CALCIUM LEVELS COMPARED	NUMBER OF PAIRS OF RATS	DIFFERENCES IN CALCIUM CONTENT BETWEEN PAIR MATES		PROBABILITY OF A FORTUITOUS OUTCOME
		Mean [1]	Standard deviation	
0.18 vs. 1.25	3	+ 0.197	0.059	0.021
0.32 vs. 1.25	3	+ 0.116	0.0065	< 0.014
0.49 vs. 1.25	4	+ 0.051	0.031	0.034

[1] The positive sign indicates that the rat on the higher calcium ration possessed on the average the higher content of calcium.

reared upon the diet containing 1.25 per cent of calcium, which was used in all three comparisons. In particular, the values obtained in the third comparison are quite uniformly low. The rates of gain in body weight of the rats in the preliminary period, presented in the fifth column of table 1, help to explain the variations in calcium content of the carcasses. There is a very evident negative correlation between rate of growth and calcium content of body. More evidence of this correlation, as well as a discussion of its significance, will be given later.

The initial calcium content of the rats may be estimated from analyses of eighteen control rats, eleven males and seven females, ranging in weight from 41 to 62 gm. and averaging 50 gm. These rats were representatives of the same litters from which the experimental rats were selected. Their average intestinal fill was 5.55 per cent and their average calcium content on the empty weight basis was 0.801 per cent. The coefficient of variation of the eighteen analyses was 7.5 per cent. No sex difference in body calcium was evident.

The calcium content of the rats at the end of the preliminary feeding period may be estimated for each group of rats reared on the same ration from the average analyses of the rats killed at that time. For the latter rats themselves, the analyses actually obtained were used. The estimated difference between final and initial calcium contents represents the calcium stores in the body, the amount of which may be compared with the calcium intake. The averages of such estimations and observations for the three ration comparisons will be found in table 3.

The ration containing 0.18 per cent of calcium induced a storage of this element averaging only 62 per cent of that effected by the ration containing 1.25 per cent of calcium. The rations containing 0.32 and 0.49 per cent of calcium permitted retentions of 76 and 87 per cent, respectively, of those brought about by the ration containing 1.25 per cent of calcium. However, the calcium intake on the lowest level of calcium was completely utilized in growth, while those of the

higher levels were utilized to the extent of 76, 55 and 25 per cent, in order. The complete utilization of the calcium on the lowest calcium ration, as well as the constant utilization with variable rates of growth on the highest calcium ration, averaging 2.38, 2.83 and 5.08 gm. per day in the three comparisons, confirm the results of Ellis and Mitchell ('33) and testify to the reality of their theory that "in the growing rat, and possibly in all growing animals, there is no integral requirement of calcium for maintenance."

The preliminary feeding evidently induced different degrees of saturation of the calcium stores, standing in relation to

TABLE 3

Summary of results of preliminary feeding period

PER CENT CA IN DIET	NUMBER OF RATS	LENGTH OF FEEDING PERIOD IN DAYS	AVERAGE GAIN IN BODY WEIGHT	AVERAGE FOOD INTAKE	AVERAGE INTAKE OF CALCIUM	AVERAGE CA RETENTION	
						In milligrams	In per cent
			gm.	*gm.*	*mg.*		
0.18	11	28	64	175	336	356	106
1.25	11	28	67	175	2336	578	25
0.32	11	28	78	192	610	465	76
1.25	11	28	79	192	2559	615	24
0.49	12	14	78	153	746	412	55
1.25	12	14	77	153	1916	476	25

one another as 62, 76, 87 and 100. The purpose of the main feeding period was to observe whether these differences in calcium saturation would induce differences in calcium retention when all rats were placed upon the ration containing the highest percentage of calcium, i.e., 1.25. The growth data of this period of uniform feeding are summarized in table 4.

No significant differences in growth were observed between pair mates except in the first comparison, in which the preliminary rations contained 0.18 and 1.25 per cent of calcium. In this comparison, in seven of the eight pairs the rat prefed on the higher calcium level grew the faster, when measured both by body weight gain and by final body length, measured from tip of nose to root of tail. The average increment in

TABLE 4

Body lengths and weights, gains in weight and food consumption for the experimental period. All rats on same ration containing 1.25 per cent Ca. All weights expressed in grams

PRELIMINARY RATION	PAIR 1		PAIR 2		PAIR 3		PAIR 4		PAIR 5		PAIR 6		PAIR 7		PAIR 8		AVERAGES	
	Low Ca	High Ca	Low Ca	High Ca	Low Ca	High Ca	Low Ca	High Ca	Low Ca	High Ca	Low Ca	High Ca	Low Ca	High Ca	Low Ca	High Ca	Low Ca	High Ca
Preliminary rations					0.18 per cent Ca vs. 1.25 per cent Ca													
Rat number and sex	3m	4m	7m	8m	9m	10m	11m	12m	13f	14f	15f	16f	17f	18f	19f	20f	...	...
Initial body weight	129	132	120	117	118	114	108	111	104	104	97	102	94	97	95	94	108	109
Final body weight	224	229	214	223	215	228	209	228	199	220	179	191	190	185	186	189	202	212
Total gain	95	97	94	106	97	114	101	117	95	116	82	89	96	88	91	95	94	103
Body length, mm.[1]	202	211	206	209	208	209	202	210	203	216	203	200	196	201	198	200	202	207
Total food	230	230	235	234	258	258	267	267	360	361	420	420	414	414	455	455	330	330
Length of test in days	16	16	18	18	18	18	17	17	28	28	41	41	36	36	40	40	27	27
Preliminary rations					0.32 per cent Ca vs. 1.25 per cent Ca													
Rat number and sex	25m	26m	29m	30m	31f	32f	35f	36f	37m	38m	39f	40f	41m	42m	43m	44m	...	...
Initial body weight	135	133	135	134	126	126	114	118	133	136	109	111	123	122	127	126	125	126
Final body weight	229	222	232	230	199	204	183	179	230	238	184	188	219	225	226	239	213	216
Total gain	94	89	97	96	73	78	69	61	97	102	75	77	96	103	99	113	88	90
Body length, mm.[1]	213	210	212	212	203	208	202	187	202	207	212	212	208	209	211	219	208	208
Total food	234	234	309	309	428	425	417	416	255	255	420	420	311	311	333	333	338	338
Length of test in days	14	14	21	21	38	38	42	42	17	17	40	40	21	21	22	22	27	27
Preliminary rations					0.49 per cent Ca vs. 1.25 per cent Ca													
Rat number and sex	55m	56m	57m	58m	59m	60m	61m	62m	63m	64m	65m	66m	69m	70m	71m	72m	...	...
Initial body weight	138	138	131	128	131	127	130	128	117	120	114	115	140	140	121	124	128	127
Final body weight	236	243	228	232	233	230	226	210	200	203	182	201	238	239	214	220	220	222
Total gain	98	105	97	104	102	103	96	82	83	83	68	86	98	99	93	96	92	95
Body length, mm.[1]	217	212	215	207	211	215	210	211	207	210	200	206	213	210	210	208	210	210
Total food	275	276	289	289	274	274	292	292	338	338	290	290	295	294	309	309	295	295
Length of test in days	18	18	19	19	17	17	21	21	31	31	26	26	16	16	22	22	21	21

[1] Measured from anus to tip of nose.

gain was 9.62 gm., and in body length, 4.75 mm.; the standard deviations of differences in gain between pair mates was 8.02 gm., and in body length, 4.76 mm. The probabilities of a fortuitous outcome are so small, 0.0078 and 0.017, that they may be neglected. It may be concluded, therefore, that subsistence upon a ration containing as low as 0.18 per cent of calcium definitely impairs growth, not only simultaneous with its consumption, but also in a subsequent period of adequate calcium nutrition. No evidence of this sort was obtained for levels of 0.32 and 0.49 per cent of calcium.

The calcium contents of the carcasses of the rats, with estimates of calcium storage involving estimates of initial calcium content based upon the analysis of rats on the same preliminary rations made at the end of the preliminary feeding period (table 1), are presented in table 5. The calcium contents of the various pair mates with different preliminary treatment are not to be distinguished from one another statistically in any of the three comparisons. Evidently the inequalities in initial calcium content induced by different levels of calcium feeding were removed during this period of uniform feeding. In agreement with this finding, the estimated calcium retentions average higher for the rats reared on the lower calcium diets in all comparisons, the average percentage increments being 19.7, 3.1 and 13.0, respectively; in milligrams, these increments average 137, 24 and 99, respectively. The standard deviations of differences in calcium storage between pair mates were 110, 124 and 164 mg. in the three comparison groups, while the probabilities of a chance outcome are 0.0066, 0.31 and 0.078, respectively. Only the first of these probabilities is small enough to neglect, so that only in the first comparison of rats pre-fed on 0.18 and 1.25 per cent levels of calcium may it be said with certainty that an initial lower saturation of the calcium stores promotes a more rapid storage of calcium in a subsequent period of uniform feeding. However, the inconclusiveness of the data in the other comparisons may have been the result of the inaccuracy in the method of estimation of the initial calcium contents of the rats, for reasons that will be developed later.

TABLE 5

Calcium contents of rats at end of experimental period with estimates of calcium storages

PRELIMINARY RATION	PAIR 1		PAIR 2		PAIR 3		PAIR 4		PAIR 5		PAIR 6		PAIR 7		PAIR 8		AVERAGES	
	Low Ca	High Ca	Low Ca	High Ca	Low Ca	High Ca	Low Ca	High Ca	Low Ca	High Ca	Low Ca	High Ca	Low Ca	High Ca	Low Ca	High Ca	Low Ca	High Ca
Preliminary rations				0.18 per cent calcium vs. 1.25 per cent calcium														
Rat number and sex	3m	4m	7m	8m	9m	10m	11m	12m	13f	14f	15f	16f	17f	18f	19f	20f	...	...
Ca in carcass, per cent, obs.[1]	0.66	0.70	0.72	0.65	0.76	0.67	0.55	0.60	0.88	0.81	0.94	0.97	0.84	0.98	0.87	0.90	0.778	0.785
Ca in carcass, mg., obs.	1494	1610	1529	1437	1640	1523	1131	1365	1759	1770	1679	1826	1589	1776	1611	1710	1554	1627
Initial Ca content, mg., est.[2]	866	1137	803	1002	789	976	719	949	691	886	642	868	621	823	628	797	720	930
Ca stored, mg., est.	628	473	726	435	851	547	412	416	1068	884	1037	958	968	953	983	913	834	697
Preliminary rations				0.32 per cent calcium vs. 1.25 per cent calcium														
Rat number and sex	25m	26m	29m	30m	31f	32f	35f	36f	37m	38m	39f	40f	41m	42m	43m	44m	...	...
Ca in carcass, per cent, obs.[1]	0.55	0.69	0.68	0.83	0.95	0.99	1.01	1.03	0.66	0.71	1.00	0.95	0.79	0.79	0.73	0.68	0.796	0.834
Ca in carcass, mg., obs.	1261	1548	1566	1894	1883	2006	1827	1842	1520	1685	1840	1799	1722	1790	1658	1677	1660	1780
Initial Ca content, mg., est.[2]	922	1058	922	1066	859	1000	774	935	908	1082	739	877	838	968	866	1000	854	998
Ca stored, mg., est.	339	490	644	828	1024	1006	1053	907	612	603	1101	922	884	822	792	677	806	782
Preliminary rations				0.49 per cent calcium vs. 1.25 per cent calcium														
Rat number and sex	55m	56m	57m	58m	59m	60m	61m	62m	63m	64m	65m	66m	69m	70m	71m	72m	...	...
Ca in carcass, per cent, obs.[1]	0.71	0.70	0.73	0.69	0.65	0.70	0.70	0.75	0.90	0.88	0.91	0.82	0.64	0.69	0.95	0.74	0.774	0.746
Ca in carcass, mg., obs.	1730	1713	1672	1633	1534	1624	1582	1583	1801	1778	1632	1630	1536	1644	2040	1612	1691	1652
Initial Ca content, mg., est.[2]	900	968	853	894	853	887	846	894	757	836	737	799	914	982	784	865	831	891
Ca stored, mg., est.	830	745	819	739	681	737	736	689	1044	942	895	831	622	662	1256	747	860	761

[1] Obs. = observed.

[2] Est. = estimated.

Fortunately, other data on the calcium retentions of the rats are available from the calcium metabolism experiments undertaken with four pairs of rats in each of the three comparisons. These data are summarized in table 6. In all of

TABLE 6

Calcium metabolism data for experimental period

RAT NO.	CALCIUM CONTENT OF PRELIMINARY DIET	EXPERIMENTAL PERIOD	CALCIUM INTAKE	CALCIUM EXCRETED	CALCIUM BALANCE
	per cent	*days*	*mg.*	*mg.*	*mg.*
3	0.18	16	2878	2102	776
4	1.25	16	2878	2313	565
7	0.18	18	2942	2160	782
8	1.25	18	2935	2244	691
9	0.18	18	3230	2340	890
10	1.25	18	3224	2498	726
11	0.18	17	3341	2580	761
12	1.25	17	3341	2594	747
25	0.32	14	2925	2255	670
26	1.25	14	2925	2416	509
29	0.32	21	3702	2996	706
30	1.25	21	3702	2964	738
31	0.32	38	4825	3816	1009
32	1.25	38	4799	3872	927
35	0.32	42	4666	3724	942
36	1.25	42	4660	4000	660
55	0.49	18	2961	2033	928
56	1.25	18	2967	2198	769
57	0.49	19	3113	2297	816
58	1.25	19	3113	2361	752
59	0.49	17	2979	2302	677
60	1.25	17	2973	2346	627
61	0.49	21	3135	2352	783
62	1.25	21	3135	2463	672

the twelve pairs of rats but one, the rat pre-fed upon the lower level of calcium retained the greater amount of calcium in the period of uniform feeding, the excess storage averaging 120, 123 and 96 mg., respectively, for the three comparison groups. The standard deviations of differences between pair mates are, in order, 75, 114 and 43 mg., and the probabilities of a fortuitous outcome, 0.035, 0.079 and 0.015. The second probability is still too large to neglect, but the whole picture is a clear demonstration of the fact that differences in the degree of saturation of the calcium stores will produce inequalities in calcium retention in a period of uniform high-calcium feeding, such that the lower the initial saturation, the more rapid the subsequent rate of retention. Throwing all of the pairs together into one statistical analysis, since the average pair differences are not statistically distinct, gives a mean difference of 113 mg., a standard deviation of 84 mg., and a probability (Student, '25) of a chance outcome of only 0.0005.

The estimations of phosphorus retentions based upon carcass analyses are irregular and offer no evidence that the previous condition of calcium (and probably phosphorus) saturation has modified the subsequent rate of phosphorus storage. However, the phosphorus balance data from the four pairs of rats in each comparison studied in this manner, suggest, in conformance with the calcium data of table 6, that the rate of phosphorus retention has been modified by the nature of the preliminary feeding. The average increments in phosphorus retention of the rats pre-fed on the high-phosphorus ration over their pair mates pre-fed on the low-phosphorus ration are, for the three comparisons, 51, 129 and 102 mg., respectively. However, none of these averages is statistically significant, the probability that chance alone could account for them being, respectively, 0.18, 0.09 and 0.10. The average ratio of retained calcium to retained phosphorus was 2.12 to 1, with no clear effect of the type of pre-feeding.

The calcium and phosphorus contents of the forty-eight rats surviving to the end of the experiment were not appreciably

affected by the different methods of pre-feeding. Hence a number of correlation studies were undertaken to throw light upon the factors modifying the content of the body in calcium and phosphorus. The product-moment correlation method of Pearson was used throughout. The correlation coefficients were calculated from the unclassified original data.

We were at first disturbed by the fact that the percentages of calcium in these rats were much lower than those reported by Sherman and Booher ('31) for rats of the same body weight on high calcium levels, the latter ranging from 1 per cent, or only slightly less, to 1.2 per cent. Our values averaged 0.785 per cent, and ranged from 0.55 to 1.03. However, our experimental rats grew at a very rapid rate, averaging 4.3 gm. daily and attaining values up to 6.7 gm. daily in individual rats. This was a much more rapid growth than that of the rats of Sherman and Booher, a fact that suggested a study of the relationship between rate of growth, as measured by average daily gain in body weight, and the calcium content of the body. Figure 1 illustrates this relationship for the forty-eight rats of the three comparisons in this experiment. The relationship is evidently a close one, the correlation coefficient being -0.929 ± 0.013, the sign meaning that the more rapid the growth, the lower the calcium content of the body. The rate of gain in weight was largely determined by the daily intake of food, the correlation coefficient for these two variables being $+0.910 \pm 0.017$.

There are two possible explanations of the inverse relationship between rate of growth and the calcium content of the body: First, that there is an inverse relationship between the rate of growth and the rate of calcification of the skeleton, and, second, that there is an inverse relationship between the rate of growth and the percentage of skeletal tissue in the body. In favor of the first explanation, Outhouse and Mendel ('33) have shown clearly that more rapid growth for rats of the same weight is associated with bones containing more water and less mineral matter. To test the second explanation the calcium contents of the carcasses of the forty-eight

rats were correlated with the ratios of calcium to phosphorus in the carcasses, which averaged 1.37 to 1. The correlation coefficient in this case is $+ 0.584 \pm 0.064$. Since this correlation is significantly positive, we may conclude that as the rate of growth increases, in response to an increasing consumption of food, the proportion of soft tissues in the body increases; conversely, a retardation of growth affects the soft tissues of the body more than the skeletal tissues. This conclusion

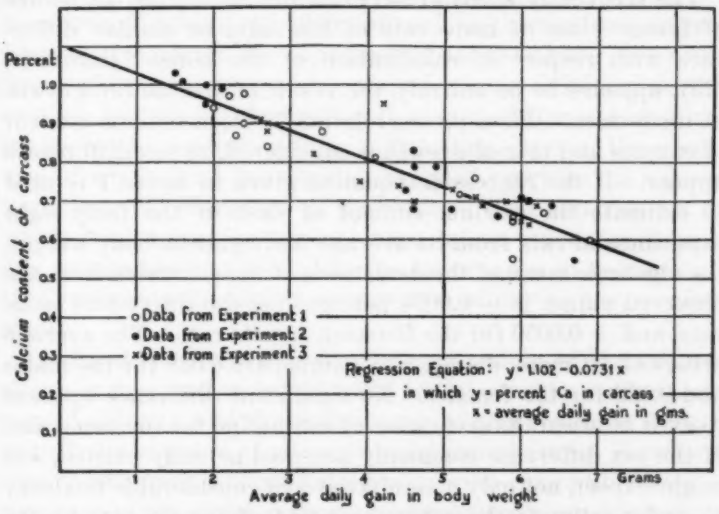

Fig. 1 Relation between the calcium contents of the bodies of the experimental rats and their average rates of growth for the previous several weeks.

is in agreement with the frequently demonstrated fact that skeletal growth is less easily retarded than the growth of the soft tissues; it is also less readily accelerated.

The calcium to phophorus ratios in the carcasses of the experimental rats ranged from 0.95 to 1 for a rat that gained an average of 6.7 gm. daily to 1.65 to 1 for a rat that gained only half as fast, the average ratio being 1.37 to 1. Assuming that 99 per cent of the body calcium is located in the skeleton (Bessey, King, Quinn and Sherman, '35) and that the ratio

of calcium to phosphorus in the skeleton is 1.94 to 1, it may be estimated that from 48 to 84 per cent of the body phosphorus, with an average of 70 per cent, was located in the bones in these experimental rats. This wide range in the proportion of skeletal phosphorus is indicative of the marked influence of the amount of food consumed on the ratio of soft tissues to bones in the body and necessarily on the proportions existing among the nutrients retained during growth.

The frequently noted greater calcium content of the bodies of female than of male rats of like age, or similar difference with respect to calcification of the bones (Hammett, '25), appears to be entirely the result of the slower growth of the female. When the correlation between calcium content of carcass and rate of growth is considered, no sex differences appear. If the regression equation given in figure 1 is used to estimate the calcium content of each of the forty-eight experimental rats from its average daily gain in body weight, the algebraic mean of the deviations of the estimates from the observed values is + 0.0024 per cent for the thirty-four male rats, and — 0.0050 for the fourteen female rats. The average errors of estimate, disregarding signs, are 0.038 for the males and 0.032 for the females. No significant difference appears to exist between the accuracies of estimation for the two sexes. If the sex difference commonly accepted actually existed, one might expect, not only a significant and considerable tendency to under-estimate the calcium content of female rats by the use of a regression equation based on male and female data, but also a greater mean error of estimate (disregarding signs) for the female rats, because the regression equation is derived from the data obtained from a group of rats consisting of thirty-four males and only fourteen females.

DISCUSSION

The bearing of these experimental findings on calcium metabolism studies, particularly those concerned with the determination of the calcium requirements of growing animals, seems clear. Since the extent to which the skeleton is saturated with respect to calcium is a determinant in the rate of calcium retention on a diet adequate in its content of this element (and presumably on any diet), calcium retentions observed under conditions of adequate nutrition are not measures of calcium requirements unless, through proper preliminary treatment, the experimental subjects have been saturated with respect to their calcium stores. Otherwise, the calcium retentions observed will exceed the actual requirements by the amount used to replete these stores, the condition of which is an expression simply of previous inadequate nutrition. Furthermore, in the evaluation of experiments in which this precaution has not been taken, more significance can reasonably be attached to the lower rates of calcium retention than to the higher as measures of day to day requirements.

In the above discussion complete saturation of the calcium stores is assumed to be the ideal initial condition of experimental subjects, and the maintenance of this condition during growth the measure of adequate calcium nutrition. However, wholly adequate calcium nutrition may be compatible with a condition of the skeleton short of complete calcium saturation. Hence, a significant problem in calcium metabolism is the determination of the lowest degree of calcium saturation compatible with maximum physiological efficiency. Conceivably this degree of saturation will be affected by the prevailing physiological condition and hence would vary with animals of different age, sex, etc.

Since the calcium content of the body and of the bones is markedly affected by the amount of food consumed as well as by its content of calcium, the control of food intake in calcium metabolism studies is essential to the most significant results.

CONCLUSIONS

1. The calcium content of growing rats is dependent upon at least two factors: a) the calcium content of the diet if the diet contains a percentage of calcium inadequate for maximum storage, and b) the rate of growth, dependent in turn largely upon the rate of food consumption. The former relation is a direct one, the latter is inverse, rapid gains being associated with low calcium contents.

2. The inverse relation between rate of growth and calcium content of body is the result of two tendencies: a) the tendency for rapid growth to be associated with slow calcification of the bones, and b) the tendency for rapid growth to be associated with high ratios of soft tissue to skeletal tissue. The growth of skeletal tissue is much more difficult to modify by feeding, either in the direction of retardation or of acceleration, than is the growth of the soft tissues.

3. The commonly accepted belief that females, as compared with males of the same age, exhibit greater percentages of calcium in their bodies and in their bones seems to be entirely referable to their slower growth.

4. Very low levels of calcium in the diet may definitely retard growth aside from their effect upon appetite, not only during the consumption of such diets, but also in a subsequent period of adequate calcium nutrition.

5. There appears to be no requirement of calcium for maintenance in the growing animal.

6. Differences in the degree of saturation of the skeletal tissues with respect to calcium, brought about by previous subsistence upon diets differing in their contents of this element, produce inequalities in the retention of calcium under uniform conditions of calcium nutrition, such that low saturation is associated with subsequent high retention of calcium.

7. It follows that the rate of calcium retention by growing animals under conditions of adequate nutrition measures the requirement of calcium only when the calcium stores have been saturated by appropriate pre-feeding. Otherwise the observed calcium retentions will be greater than the day to day requirements of calcium.

8. Since the calcium content of growing rats (and presumably of other animals) and the extent of calcification of their bones, is dependent upon the amount of food consumed, as well as upon its mineral content, the control of the food intake of experimental animals in calcium nutrition studies is essential to the greatest accuracy of all comparisons made and to the greatest significance of the conclusions deduced.

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A STUDY OF THE DIETARY FACTORS CONCERNED IN NUTRITIONAL MUSCULAR DYSTROPHY [1]

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I. CAUSATION AND PREVENTION OF NUTRITIONAL MUSCULAR DYSTROPHY

The experiments of Goettsch, Pappenheimer and their collaborators (Goettsch and Brown, '32; Goettsch and Pappenheimer, '31; Pappenheimer and Goettsch, '31, '34 a, '34 b; Rogers, Pappenheimer and Goettsch, '31; Victor, '34) showed, for the first time, that muscular dystrophy could be produced in laboratory animals (guinea pig, rabbit and duckling) by nutritional means. The similarity between the histological and clinical pictures obtained with these animals and those found in cases of progressive muscular dystrophy suggested that the experimental condition in animals might be closely related to the clinical condition found in man. In view of this possible relationship it was deemed worthwhile to investigate more closely rabbits affected with nutritional muscular dystrophy, and a series of experiments were planned with this in mind.

The control diet

Our control animals were maintained on a standard diet [2] which furnished an adequate supply of vitamins A, B, E and G, and a small amount of D. The rabbits on this diet invariably made a steady gain in weight until maturity was

[1] The data presented in this paper are taken from the dissertation submitted by Howard C. Spencer to the Graduate College of the University of Nebraska, January, 1936, in partial fulfillment of the requirements for the degree of doctor of philosophy.

[2] Rabbit chow obtained from the Ralston Purina Company.

reached, appeared to be in excellent physical condition, and served as controls for the animals on the experimental diets.

Experiments with original dystrophy producing diet 13

At the outset of our experiments it was deemed advisable to produce dystrophy in rabbits by means of the diet used originally by Goettsch and Pappenheimer ('31), their diet 13, to establish definite criteria by which the manifestations of dystrophy could be recognized in the experimental animals. Furthermore, since this diet was to be used as the basic ration in the study of the factors involved in the production of dystrophy, it was of paramount importance to have a record of the behavior of rabbits on diet 13.

This diet was made by mixing 355 parts rolled oats,[3] 180 parts wheat bran,[4] 75 parts casein, 80 parts lard, 10 parts cod liver oil, 10 parts NaCl, and 15 parts $CaCO_3$. To one such batch a mixture of 50 cc. 20 per cent $FeCl_3$ and 100 cc. ether was added in a closed vessel and allowed to stand, with frequent shaking, for about ½ hour. The food was then spread out in shallow trays and left over night to allow the ether to evaporate. To this was now added 275 parts of skim milk powder and mixed thoroughly in a mechanical mixer. The food was prepared fresh at least once a week. Since rabbits are resistant to scurvy, orange juice was not administered.

A number of animals were maintained on this diet 13 and all became dystrophic. The average time required by the rabbits (about a month old and weighing about 500 gm.) to attain maximum weight was 26 days, while the average number of days on diet 13 before death or at any rate before a moribund condition developed was 34 days (28 to 43 days). This corresponds fairly well with the range of 14 to 50 days, which Goettsch and Pappenheimer ('31) found necessary to produce definite dystrophy on this diet. Furthermore, their description of the appearance and behavior of the dystrophic rabbits agrees well with our own observations. Histological

[3] Quaker brand.
[4] Pillsbury.

examination of the dystrophic muscles revealed essentially the conditions described by Goettsch and Pappenheimer ('31). The clinical observation of the rabbit and, whenever possible, histological examination of the muscle were the means used to ascertain the extent of dystrophy. When the progress of the disease or of the recovery was followed histologically, biopsy material was removed aseptically from the gluteus maximus under novocaine anesthesia.

Experiments on the influence of ferric chloride

Since the most essential feature in the preparation of diet 13 is the treatment with the ethereal $FeCl_3$, it seemed desirable to investigate the effect of this factor upon the dystrophy producing property of the food. For this purpose experiments were made with three different diets:

a. Diet 11 (untreated diet 13).
b. Diet 207 (diet 13 treated with aqueous $FeCl_3$).
c. Diet 201 (control diet treated with ethereal-$FeCl_3$).

a. Diet 11 is prepared exactly like the diet 13 with the omission of the treatment with ethereal ferric chloride and, therefore, feeding this diet should give some indication of the role of ferric chloride in the production of dystrophy. Furthermore, since the ethereal-ferric chloride treatment was originally designed to destroy vitamin E (Waddell and Steenbock, '28), its omission makes diet 11 a fairly good source of this vitamin, and feeding experiments with it should throw light on the effectiveness of vitamin E in preventing dystrophy.

Goettsch and Pappenheimer ('31) report that rabbits react to this diet 11 exactly as they do to diet 13. Victor ('34), in a later publication, cites cases of rabbits becoming dystrophic on diet 11 as early as the twenty-third day and as late as the three hundred and thirty-first day. In our experiments, three rabbits maintained on this diet showed no signs of dystrophy at the end of a period of 69 to 83 days, which does not bear out the conclusion of Goettsch and Pappenheimer that both diet 11 and diet 13 are equally dystrophic

in their effect. Owing to lack of time, these three rabbits were shifted to diet 13, and in every case dystrophic manifestations, confirmed by histological examination, set in very soon afterward. These results indicate that diet 11 at least induces a latent dystrophic condition or in some way prepares the organism for the rapid break down which comes with the change to diet 13.

A fourth rabbit was maintained on diet 11 for 210 days without showing the slightest signs of the disease. A biopsy performed on a fifth rabbit after 121 days on diet 11 (weight 2840 gm.) showed that the muscles were entirely normal, but a second biopsy taken at the end of 212 days showed definite lesions in the muscles. In all probability the dystrophic condition developed about the one hundred and forty-seventh day, when there was a break in the body weight. However, since this animal lived 210 days on diet 11, it must have been suffering from a very mild or chronic form of dystrophy.

In contrast to this, a sixth rabbit became definitely dystrophic in 42 days on diet 11, and its condition was corroborated histologically.

In general, our results agree with those of Victor ('34), showing great variability in the time of onset, in the severity and the progress of the disease in animals maintained on diet 11. But in spite of these wide variations it can be stated that neither the presence of the ferric chloride itself in diet 13 nor the absence of vitamin E from this diet can be regarded as the principal factor in the production of nutritional muscle dystrophy.

b. Waddell and Steenbock ('28) have shown that ethereal-ferric chloride treatment destroys vitamin E in synthetic diets, while an aqueous solution of $FeCl_3$ fails to do so. For this reason we decided to compare the effect of the regular diet 13 with that of a diet treated with aqueous-ferric chloride. Our diet 207 is therefore like diet 13 except for the fact that it had been treated with aqueous-ferric chloride. The use of a water solution, of course, necessitates a longer time for evaporation. The diet tends to sour more quickly than diet 13 and was made up fresh at more frequent intervals.

The animals on this diet 207 behaved exactly like those on diet 13. Apparently the water solution of $FeCl_3$ is as effective as the ether solution in rendering the diet dystrophy producing. This bears out our conclusion from the experiments with diet 11 that the destruction of vitamin E cannot be the principal factor in diet 13 for its dystrophy producing action.

c. We studied the influence of the ethereal-ferric chloride treatment further by treating the control diet in the same manner as we treated diet 13. This diet 201 contained the same quantity of $FeCl_3$ as the original diet 13, and since the treatment destroyed the vitamin E and much of the vitamin A content of this food, it seemed that feeding it to rabbits would help to ascertain the effect of the $FeCl_3$ per se as well as that of a diminished vitamin E (and vitamin A) supply. We fed this diet 201 also supplemented with potent sources of vitamin E or vitamin A, as well as of a combination of these vitamins.

Wheat germ oil was used as a source of vitamin E, and throughout this report E will be used to designate wheat germ oil. This was a cold pressed oil, 2 cc. of which were equivalent to 100 gm. of wheat germ. The animals receiving vitamin E were given 10 to 12 drops of this oil daily by pipette. This level of wheat germ oil is somewhat higher than that used by Goettsch and Pappenheimer ('31).

An oil solution of carotene was used as a source of vitamin A, and this had a biological potency of not less than 7500 new U. S. P. units of vitamin A per gram. Five drops of this solution were administered daily to the rabbits receiving the vitamin A supplement. In this report A will always refer to this preparation. The vitamin preparations were kept in well-stoppered bottles in the ice box.

Six animals were maintained on this diet 201 for at least 200 days and none of them showed any dystrophic manifestations. Some of the rabbits (two) did not grow so well as those fed the untreated control diet, but the poorer growth may have been due to respiratory infections from which these animals suffered.

The results with diet 201 indicate that the presence of $FeCl_3$ by itself does not render a diet unsuitable for rabbits. Furthermore, the ethereal-ferric chloride treatment apparently does not destroy the principal factors which prevent the onset of dystrophy.

From the experiments on feeding diets 11, 207 and 201 we may conclude that: 1) Nutritional muscle dystrophy is not caused by the ferric chloride present in the diet. 2) The destruction of vitamin E is not the principal cause in making the diet dystrophy producing.

Substitution of a vegetable oil in diet 13

Nutritional encephalomalacia is produced in the chick by a synthetic diet similar to diet 11. Pappenheimer and Goettsch ('34 b) found that the substitution of certain vegetable oils for the lard in the original diet provides adequate protection against this disorder. We thought it advisable to test the effect of modifying diet 13 by the substitution of a vegetable oil[5] for the lard, but our rabbits became dystrophic on this modified food as quickly, if not more quickly, than on diet 13.

Diet 13 supplemented with dry alfalfa and alfalfa extracts (diets 220 to 252)

Simultaneously with the feeding experiments described above we carried out curative experiments. One of the first definite cures was effected by feeding fresh green alfalfa together with diet 13. The work with curative diets will be the subject of the next section, but we shall discuss here some of the experiments with alfalfa because they focused our attention upon this as a possible source of the factors active in preventing dystrophy. We investigated the effectiveness of dry alfalfa and of extracts prepared from both fresh and dry alfalfa in preventing nutritional muscle dystrophy. For the extracts, we used a kilogram of finely ground fresh, green alfalfa or dry alfalfa. This ground material was pressed,

[5] Mazola.

extracted with water by percolation, again pressed and re-extracted. The total aqueous extract was made up so that 3 liters were equivalent to 1 kg. of the original alfalfa. The extracts were preserved in the ice box.

The residue after the two extractions with water and with alcohol was dried and kept in a closed container.

The extracts of alfalfa did not afford any protection against the development of muscle dystrophy in rabbits fed on diet 13. Since the dry alfalfa itself is not protective, the extracts of dry alfalfa would not be expected to exert any influence either. But the fresh, green alfalfa is definitely a protective agent, and it might be expected that one or the other of the extracts, or their combination, would also prove protective. Our method of extraction did not exclude ferment action, and it is not impossible that the necessary factors might have been destroyed during the extraction. The effect of the ferric chloride treatment and the fact that dry, well-cured alfalfa is lacking in protective action suggest that the protective factor or factors are probably easily destroyed.

On diet 220, which is diet 13 supplemented with dry alfalfa, all the rabbits became dystrophic, though the onset of the disease was slower than on diet 13 alone. The earliest onset was after 55 days on this diet and some were still normal even after 100 days.

We are led to conclude that the factor or factors present in green alfalfa, which can prevent the onset of dystrophy, is destroyed by the curing process as well as by the extraction methods employed by us, and that it is an unstable substance.

Experiments with diet 13 and lettuce

In our attempt to determine the effectiveness of green foodstuffs in preventing dystrophy we performed experiments feeding diet 13 together with fresh, green lettuce. The lettuce was selected for this purpose because it could be procured fresh daily and because it has a high vitamin E content. We were careful to select only the fresh, green leaves for feeding, a total of 50 gm. per day being fed to the rabbits in two portions during the day.

All the rabbits maintained on diet 13 supplemented daily with 50 gm. of lettuce became dystrophic, the time of onset of the disease varying from 30 to 100 days, or 53 days on the average. The feeding of lettuce has thus prolonged the period during which a rabbit could be maintained on diet 13 but did not prevent the development of dystrophy. In this respect, therefore, the fresh, green lettuce differed decidedly from fresh, green alfalfa.

Experiments with diet 13 and vitamin supplements

The curative effect of green alfalfa and the instability of the active factor has made the further study of vitamin supplements desirable. Although diet 13 is deficient in vitamin E, the experiments previously described indicate that avitaminosis E is not the principal factor in the production of dystrophy. The following diets were devised to study this problem further:

a. Diet 204 (diet 13 supplemented with whole wheat germ).
b. Diet 213 (diet 13 supplemented with whole wheat germ treated with ethereal ferric chloride).
c. Diet 214 (diet 13 supplemented with wheat germ oil).
d. Diet 215 (diet 13 supplemented by carotene, or by a combination of carotene and wheat germ oil).
e. Diet 216 (diet 13 supplemented by a combination of wheat germ oil and lettuce, or by a combination of wheat germ oil and dry alfalfa).

a. Diet 204. Whole wheat germ being one of the most potent sources of vitamin E, a diet was made up by mixing 100 gm. of diet 13 with 20 gm. of fresh wheat germ. Rabbits fed this diet were completely protected from dystrophy. However, this does not necessarily mean that vitamin E is the protective agent as some other constituent of the germ may be responsible for the prevention of the dystrophy. Previous experiments have shown that, at least, vitamin E alone is not the preventive factor. The recovery experiments, reported in the next section, must be considered in this connection, since they furnish further evidence of both the preventive and curative action of wheat germ.

In an effort to determine the nature of the effective agents present in wheat germ we performed two sets of experiments. One was concerned with the effect of ethereal-ferric chloride treatment of whole wheat germ, while the other dealt with the effectiveness of wheat germ oil in preventing dystrophy.

b. Diet 213. This diet was like diet 204 except that the whole wheat germ used to supplement the diet 13 had been treated with ethereal-ferric chloride. The results obtained with this diet show that the rabbits were probably maintained for a longer time before becoming dystrophic than on the straight diet 13, although eventually they all became dystrophic. Obviously, the treatment with ethereal-ferric chloride has destroyed or rendered ineffective the protective factor in the wheat germ.

c. Diet 214. Since the vitamin E of wheat germ is found in the wheat germ oil, feeding diet 13 supplemented with this oil should throw light on the role of vitamin E in the prevention of dystrophy as well as help to determine the nature of the protective agent in the whole wheat germ.

The results of these experiments show that the administration of wheat germ oil does not affect the dystrophy producing action of diet 13, except that the onset of the disease is somewhat delayed. Three of the rabbits became definitely dystrophic in 35 to 41 days, one rabbit in 58 days, while another rabbit showed only mild symptoms even after 100 days on this diet. The average time for the onset on diet 214 was 55 days, which is somewhat longer than on diet 13 alone. These experiments with diet 13 supplemented with wheat germ oil corroborate the conclusion that the absence of vitamin E is not the principal factor in the production of muscle dystrophy.

The results with diets 204, 213 and 214 lead, therefore, to the following conclusions: 1) Wheat germ contains a factor which prevents the development of muscle dystrophy in rabbits. 2) This protective factor is destroyed by ethereal-ferric chloride treatment. 3) Cold pressed wheat germ oil does not contain the protective factor necessary to prevent dystrophy.

d. Diet 215. Inasmuch as wheat germ oil was found to be ineffective, while whole wheat germ itself was very effective in the prevention of dystrophy, it seemed reasonable to suppose that a combination of vitamin E with some other factor acts as the protective agent. The work of Aberle ('34) suggests that avitaminosis A may play a part, and we, therefore, arranged a series of experiments with diet 13 supplemented by carotene or by a combination of carotene and wheat germ oil. Since all the rabbits on this modified diet became dystrophic, it is obvious that neither carotene alone nor a combination of carotene with wheat germ oil furnish protection against dystrophy. Experiments in which the diet 13 was supplemented with the vitamin B complex (fresh brewers' yeast) have likewise failed to offer any protection against the development of dystrophy.

The results obtained on diet 13 with various vitamin supplements lead to the conclusion that the dystrophic condition in rabbits on this diet is not due to a deficiency of either vitamins A, B or E, or a combination of vitamins A and E.

e. Diet 216. We have shown previously that wheat germ or fresh, green alfalfa furnish adequate protection against nutritional muscle dystrophy, but dry alfalfa, fresh green lettuce, carotene, wheat germ oil, yeast or a combination of carotene and wheat germ oil are entirely ineffective. In the case of dry alfalfa, lettuce or wheat germ oil the onset of the disease was, however, somewhat delayed as compared with diet 13 unsupplemented by any of these products. These findings suggested that possibly a combination of the various supplements might prove effective and subsequent feeding experiments showed that either a combination of lettuce with wheat germ oil or of dry alfalfa with wheat germ oil is effective in preventing the development of dystrophy in the rabbit. These experiments gain more significance when they are considered in conjunction with the study of curative diets, discussed in the next section, but they definitely point to the conclusion that there are at least two factors concerned in the prevention of dystrophy. Both factors are furnished by

whole wheat germ or by fresh, green alfalfa. On the other hand, one must be found in dry alfalfa or in fresh lettuce while the other is present in the wheat germ oil (cold pressed).

The experiments on the causation and prevention of muscle dystrophy were conducted simultaneously with curative studies. The experiments with the curative diets corroborate the findings reported in this section. A general summary will, therefore, be given at the conclusion of the second section.

II. THE CURE OF NUTRITIONAL MUSCULAR DYSTROPHY

The recovery experiments to be reported here were all carried out according to the same general plan. Muscular dystrophy was developed in rabbits by one of the causative diets, and recovery was attempted by changing the animal to another diet. Whenever this was possible, the state of dystrophy as well as the recovery from dystrophy was checked histologically, biopsies having been performed at suitable stages during the experiment.

Although the results of the microscopic study of the muscles will be presented in a separate paper, it may be pointed out here that, whenever the dystrophic condition was cured by a change in the diet, not only have the outward manifestations characteristic of the behavior of the diseased rabbits disappeared but the muscles themselves have undergone progressive regeneration with ultimate restoration of the normal histological structure.

Experiments with control diet

When the question was first posed whether or not the recovery from dystrophy could be effected, it was decided to place diseased rabbits on an adequate food such as our control diet. Since two dystrophic rabbits made a rapid and complete recovery on this diet, we were convinced that a dystrophic rabbit could be cured of the disease when placed on a proper diet. Naturally, recovery can no longer be expected in animals in which the dystrophy has already progressed

so far that it is unable to eat. The preliminary trials with the control diet led us to secure recovery with the basic diet 13 supplemented with the proper sources of the protective factors.

Diet 13 and green alfalfa

Supplementing diet 13 with 50 gm. of fresh, green alfalfa daily brought about definite recovery from muscular dystrophy, as can be seen from the results recorded in table 1.

TABLE 1

Experiments with diet 13 and green alfalfa as a recovery diet

RABBIT	DIET	DIET SUPPLEMENT	BODY WEIGHT IN GRAMS			NUMBER OF DAYS ON DIET		BIOPSY	REMARKS
			At beginning of experiment	Maximum weight attained	At close of experiment	To reach maximum weight	During decline in weight		
213	11	—	660	2310	2310	83	0	—	Apparently normal
	13	—	2310	2400	2150	2	5	—	Dystrophic
	13	Lettuce and green alfalfa	2150	2360	2360	30	0	—	Apparently entirely recovered
328	13	—	400	870	840	37	3	I	Dystrophic (++)[1]
	13	Green alfalfa	840	980	980	8	0	II	Definitely improved but still dystrophic (+)
	13	Green alfalfa	980	1890	1890	60	0	—	Apparently entirely recovered

[1] The degree of dystrophy is indicated by the number of plus signs and is based on the estimate of the degenerative process from a microscopic study of the affected muscles.

Diet 13 and lettuce

Encouraged by the recovery effected on diet 13 supplemented with green alfalfa, it seemed advisable to test the effectiveness of other green foodstuffs. We selected for this purpose green lettuce but the results were disappointing and revealed that lettuce alone added to the dystrophic diet 13 does not effect a cure from this condition. These results

substantiated the earlier findings that green lettuce, unlike the green alfalfa, does not contain the necessary factor to prevent the development of muscle dystrophy.

Diet 13 with lettuce and wheat germ oil

Since in the earlier work we found that a combination of lettuce and wheat germ oil was effective in preventing the onset of dystrophy, we studied this combination further by means of curative experiments. The results of these experiments are recorded in table 2. Rabbits 296 and 297 became dystrophic on diet 13 and lettuce, while rabbits 299 and 300 developed dystrophy on diet 13 and wheat germ oil. However, all four animals made rapid recovery when wheat germ oil was added to the former, or lettuce was added to the latter. These results corroborate the previous findings and substantiate further our conclusion that two factors are necessary either for prevention or cure of muscle dystrophy in the rabbit, one factor being furnished by foods like lettuce and the other by wheat germ oil.

Diet 13 and whole wheat germ

To check the results previously described of preventing dystrophy on diet 13 supplemented with whole wheat germ, we performed a series of curative experiments with this substance. The results reported in table 3 show that wheat germ is equally effective both as a preventive and as a curative food. Whole wheat germ, therefore, supplies all the necessary factors for preventing or for curing muscle dystrophy.

Diet 13 with dry alfalfa and wheat germ oil

As a corollary to the earlier experiments, we tried the addition of wheat germ oil to diet 220 (diet 13 supplemented with dry alfalfa) as a curative measure. The results with this diet are summarized in table 4. Rabbit 255 became dystrophic on diet 220, but upon the administration of wheat germ oil it rapidly recovered from the disease. Rabbit 288 developed

TABLE 2

Experiments with diet 13 supplemented with lettuce and wheat germ oil (E) as a curative diet

RABBIT	DIET	DIET SUPPLEMENT	BODY WEIGHT IN GRAMS			NUMBER OF DAYS ON DIET		BIOPSY	REMARKS
			At beginning of experiment	Maximum weight attained	At close of experiment	To reach maximum weight	During decline in weight		
280	248	—	560	1500	1230	32	14	—	Pronounced dystrophy
	13	Lettuce	1230	Almost constant weight throughout period		28		—	Still definitely dystrophic
	13	Lettuce and E	1230	2300	2300	91	0	I	Apparently entirely recovered (corroborated histologically)
296	13	Lettuce	500	1690	1600	47	1	—	Dystrophic
	13	Lettuce and E	1600	1700	1620	4	2	—	Apparently improving then sudden paralysis (+)
297	13	Lettuce	500	1040	900	25	6	—	Dystrophy and paralysis
	13	Lettuce and E	900	900	760	0	9	I	Eating and showing improvement. Dystrophic (+++)
	13	Lettuce and E	760	1800	1800	49	0	II	Definite but not complete recovery
	13	Lettuce and E	1800	2120	2120	108	0	III	Practically complete recovery
299	13	E	500	1510	1470	38	3	—	Dystrophic
	13	Lettuce and E	1470	1660	1400	7	3	—	Died—diarrhea. Was making rapid recovery
300	13	E	600	1470	1300	33	5	I	Dystrophic (++)
	13	Lettuce and E	1300	1960	1960	49	0	II	Great improvement but not complete recovery
	13	Lettuce and E	1960	2800	2800	97	0	III	Practically entirely recovered
302	13	A and E	520	1500	1430	38	3	—	Dystrophic
	13	Lettuce and E	1430	1530	1530	6	0	—	Apparently improving then sudden paralysis (++)

TABLE 3

Experiments with diet 13 and whole wheat germ (diet 204) as a curative diet

RABBIT	DIET	DIET SUPPLEMENT	BODY WEIGHT IN GRAMS			NUMBER OF DAYS ON DIET		BIOPSY	REMARKS
			At beginning of experiment	Maximum weight attained	At close of experiment	To reach maximum weight	During decline in weight		
291	13	—	600	1350	1310	28	3	I	Dystrophic (+)
	13	Wheat germ	1310	2400	2250	55	8	II	Definite recovery (corroborated histologically)
294	13	—	550	1270	1070	26	3	I	Dystrophic (+)
	13	Wheat germ	1070	2060	2060	40	0	II	A good, although not complete recovery
	13	Wheat germ	2060	2900	2900	95	0	III	Complete recovery

TABLE 4

Experiments with diet 13 and dry alfalfa (diet 220) and wheat germ oil (E) as a curative diet

RABBIT	DIET	DIET SUPPLEMENT	BODY WEIGHT IN GRAMS			NUMBER OF DAYS ON DIET		BIOPSY	REMARKS
			At beginning of experiment	Maximum weight attained	At close of experiment	To reach maximum weight	During decline in weight		
255	220	—	370	1750	1480	47	8	I	Dystrophic (+++)
	220	E	1480	2250	2250	30	0	II	Definitely improving but still dystrophic (+)
	220	E	2250	3180	3180	135	0	—	Apparently entirely recovered
288	13	A and E	650	1860	1680	43	6	I	Dystrophic (++)
	220	E	1680	1980	1450	9	8	—	Apparently slight recovery—then violent onset of dystrophy and sudden paralysis (++++)

dystrophy on diet 13 supplemented with carotene and wheat germ oil. At that stage it was placed on diet 220 with wheat germ oil and showed rapid improvement for 9 days. However, this animal then began to lose weight and within 8 days it suddenly became paralyzed and died. Since this rabbit showed marked recovery for the first 9 days on this diet, the subsequent paralysis and death do not necessarily reflect upon the effectiveness as a curative diet of diet 220 and wheat germ oil.

The results with rabbit 255 again clearly point to the need of two factors for the cure of dystrophy, one of which is supplied by dry alfalfa and the other by wheat germ oil.

SUMMARY AND CONCLUSION

1. Muscle dystrophy in the rabbit could not be prevented by the addition of the following supplements, singly, to diet 13: Dry alfalfa, a vegetable oil, lettuce, vitamin A (carotene in oil), vitamin E (cold pressed wheat germ oil), or vitamin B (yeast).

2. Neither the omission of the ethereal-ferric chloride treatment nor the substitution of aqueous-ferric chloride treatment of the basic diet entirely abolished its dystrophy producing effects.

3. Prevention of muscle dystrophy as well as cure of the dystrophy already developed was effected by feeding the following supplements along with the dystrophic diet 13: Fresh green alfalfa, lettuce and vitamin E (wheat germ oil), dry alfalfa and vitamin E (wheat germ oil) or whole wheat germ.

The experimental results lead to the conclusion that there must be at least two factors involved in the prevention or in the cure of muscle dystrophy. Both factors are present in fresh green alfalfa or in whole wheat germ. On the other hand, one of these factors is supplied by wheat germ oil (cold pressed), while the other is present in lettuce or in dry alfalfa. At least one of the factors is easily destroyed by ethereal-ferric chloride, by drying or by extraction with water or alcohol.

NOTE ON THE OCCURRENCE OF PARALYSIS

Occasionally in the course of an experiment an amyotonic condition suddenly developed in our animals which resembled flaccid paralysis. This condition was characterized by sudden onset, very rapid progress and usually fatal termination.

This peculiar paralytic condition developed in some of our rabbits on diet 13 alone, on diet 13 with wheat germ oil, diet 13 with carotene and wheat germ oil, diet 13 with lettuce and wheat germ oil, and on diet 13 with dry alfalfa and wheat germ oil.

In rabbits 285, 286, 290 and 304, which were on a dystrophy producing diet, the paralysis apparently developed independently of the dystrophy. Microscopic examination of the muscles showed advanced lesions in rabbits 285 and 286, while in rabbits 290 and 304 only the earliest signs of dystrophy were present, but the paralysis in all four animals was marked and severe. Thus, the severity of the muscle dystrophy and that of the paralysis did not run parallel. The paralysis, furthermore, resembled in no way the dystrophic condition outwardly, and the affected animals assumed a sprawling position with their limbs flaccid and extending sidewise.

In some animals (rabbits 296 and 302) the paralysis appeared while they were recovering from a previous attack of dystrophy. These animals had developed definite dystrophy and were shifted to a curative diet, on which they already were making marked improvement. The microscopic examination of their muscles actually revealed regenerative recovery when they were suddenly stricken by the paralysis.

Rabbit 297 on diet 13 supplemented with lettuce became dystrophic and paralyzed at the same time. This animal was still able to feed and recovered ultimately when wheat germ oil was added to the above diet. Three biopsies were performed on this animal during its recovery, which fully corroborated the findings from observation of the animal's behavior.

Ringsted ('35) described paresis appearing in rats suffering from chronic avitaminosis E which is quite similar to the neuropathic disturbance noted in vitamin A deficient rats by Aberle ('34). The paresis studied by Ringsted developed in adults, was of the relaxed type and was accompanied by disturbances of deep-seated sensibility much more than of the cutaneous sensibility of the legs and tail. Aberle did not observe changes in sensibility or atrophy of the skin and fur, as was the case in Ringsted's animals.

The variety of diets upon which paralysis developed in our rabbits makes it very difficult to assign its causation to any particular factor. The one thing common in all our cases is the use of diet 13; however, only a small proportion of the rabbits on this diet or variations of this diet have actually become paralyzed.

Our findings seem to point out that whatever may have been responsible for paralysis bears no direct relation to the causation of muscle dystrophy. The paralytic condition occurred sporadically in animals either affected by dystrophy or while they were recovering from the dystrophy, and is in all probability an entirely unrelated disorder.

The fact that paralysis occurred only among the rabbits from three litters, which were obtained from the same rabbitry, suggests the possibility that we are dealing with an hereditary disability. The extreme suddenness of the onset of the paralysis and the rapidity with which it usually terminated in death made it impossible to study the paralyzed animals systematically.

Summary of Feeding experiments

Causative diets		*Preventive diets*	*Curative diets*
Onset of dystrophy			
Rapid	*Slow*		
Diet 13	Diet 11 (untreated diet 13)	Rabbit chow	Rabbit chow
Diet 13 treated with aqueous-ferric chloride	Diet 13 + wheat germ treated with ethereal-ferric chloride	Diet 13 + wheat germ	Diet 13 + wheat germ
Diet 13 with mazola instead of lard	Diet 13 + E	Diet 13 + lettuce + E	Diet 13 + green alfalfa
Diet 13 + alfalfa extracts	Diet 13 + E + A	Diet 13 + lettuce + E + A	Diet 13 + lettuce + E
Diet 13 + yeast	Diet 13 + lettuce	Diet 13 + dry alfalfa + E	Diet 13 + dry alfalfa + E
Diet 13 + A	Diet 13 + dry alfalfa	Rabbit chow treated with ethereal-ferric chloride (diet 201)	
		Diet 201 + E	
		Diet 201 + E + A	

E = cold pressed wheat germ oil, A = carotene in oil.

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CHANGES IN THE VAGINAL EPITHELIUM OF THE RAT ON AN EXCESSIVE VITAMIN A DIET[1]

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Many investigators have reported that excessive cornification, in the vaginal epithelium of the albino rat, is an early manifestation of vitamin A deficiency, Evans and Bishop ('22), Baumann and Steenbock ('32), Aberle ('33), Mason and Ellison ('35).

Evans ('28) noticed that four-fifths of the copulations were unsuccessful due to a lack of proper implantation. He also reported that only about one-fifth of the copulations lead to the birth of litters.

Sherwood, Toth and Carr ('34), Sherwood and Luckner ('35) reported a change in the colloidal content of the thyroid gland and a change in the histological picture when large amounts of cod liver oil, haliver oil and, carotene were administered. It was therefore concluded that the excessive vitamin A added to the normal diet was responsible for a greater activity of the epithelial tissues.

EXPERIMENTAL PROCEDURE

Since the vaginal epithelium was altered as reported by Mason and Ellison ('35) when a diet deficient in vitamin A was administered, it was deemed of sufficient interest to investigate the effect of large amounts of vitamin A on the vaginal tissues.

[1] The carotene used in the present investigation was generously furnished by S. M. A. Corporation, Cleveland, Ohio.

The technic of Long and Evans ('22) was used in the study of the vaginal smear picture. The source of vitamin A was carotene. Corn oil as well as cottonseed oil was used for the control animals. Since cottonseed oil is used as a base for carotene, it was thought necessary to investigate the effect of this oil in order that the results could be interpreted as being due to the carotene or provitamin, and not to the oil.

A group of eighteen animals, 150 days of age, were examined at 8-hour intervals for the normal vaginal smear content until eight complete oestrous cycles had been determined. These animals were then placed on 1500 international units of carotene daily, in addition to the normal diet, for a period of 15 days. The vaginal smear technic was continued during the carotene administration. Vaginal smears were examined after the carotene was discontinued until eight normal oestrous cycles had again been observed.

A second group of sixteen albino rats, 150 days of age, were used and the procedure repeated as mentioned above with the exception that 3750 international units of carotene were given daily for a period of 15 days.

Both experimental and control animals were fed dried skim milk, dried meat, cod liver oil, wheat germ, yellow corn grits, black-strap molasses, iodized salt, organic minerals, wheat cereal, oat cereal, corn cereal and vegetable fiber. This material was given in the form of a stock dog food[2]. This diet was supplemented with fresh whole milk and green lettuce.

RESULTS

The control animals showed no change in the vaginal smear even though they had received 0.5 cc. of cottonseed oil daily for a period of 15 days. The rats that were administered corn oil did not show a change in the vaginal picture. These rats demonstrated an oestrous cycle of approximately 4½ days in every case.

[2] Purina dog chow obtained from the Purina Co., St. Louis.

Table 1 represents the averages of both control and experimental animals. Five individual animals of each experimental group are also presented for the purpose of showing animal variations. The vaginal smears were observed to be abnormal within 2 days following carotene administration. The smears did not progress from the nucleated epithelial to the cornified cell stage.

TABLE 1

The effect of vitamin A given in the form of carotene on the vaginal smear picture

RAT NO.	NORMAL CYCLES	AVERAGE DAYS	DAYS ABNORMAL	NORMAL CYCLES	AVERAGE DAYS	REMARKS
1	8	4.5	32	7	4.5	Animals 1–5 1500 international units daily for 15 days
2	4	4.4	33	5	4.5	
3	6	4.5	34	4	4.6	
4	6	4.5	34	5	4.7	
5	7	4.4	34	5	4.6	
6	8	4.5	34	8	4.4	Animals 6–10 3750 international units daily for 15 days
7	8	4.5	31	5	4.0	
8	5	4.5	31	5	4.0	
9	5	4.5	31	5	4.4	
10	7	4.5	31	5	4.2	
11	5	4.2	—	5	4.2	Cottonseed oil controls
12	5	4.2	—	5	4.2	
Average of animals on 1500 units of vitamin A—18 rats						
	7	4.3	34	6	4.5	
Average of animals on 3750 units of vitamin A—16 rats						
	6	4.4	31	5	4.2	

Upon examination of the vaginal smears an excess of nucleated epithelial cells were seen, regardless of the phase of the oestrous cycle. Leucocytes were also observed continuously but were not as numerous as were the epithelial cells. Occasionally a few scattered cornified cells were observed which might have been obtained from the orifice of the vagina.

The normal oestrous cycle was not observed for approximately 20 days after the carotene feeding had been discontinued.

DISCUSSION

Baumann and Steenbock ('32) observed dioestrous smears within 1 week and oestrum in 2 weeks after the administration of carotene to rats that had been held on a diet deficient in vitamin A. The rapid return to normal seems to indicate a powerful vitamin effect on the growth of epithelial tissues.

The results obtained in the present investigation seem to be due to a rapid cell growth as indicated by the large number of young nucleated epithelial cells which predominated throughout the experimental phase of the problem.

The carotene must have been converted into vitamin A in excessive amounts since the rapid growth was noticed within 2 days after experimentation had begun.

Since the control rats gave no indication of a changed vaginal smear picture, it is clearly demonstrated that the change must have been brought about through the effect of large amounts of vitamin A in the experimental animals. This phenomenon must be expected since vitamin A has an effect on the growth processes of the animal in general. However, it is not to be expected that any normal growing process would cause a change in the oestrous cycle as abnormal as reported in the present investigation.

Further studies are being made for the purpose of finding a possible effect elsewhere in the organism which might be directly or indirectly responsible for the changes observed in the vaginal smears. The animals did not show a desire to copulate in most instances. When the animals did copulate, no pregnancy resulted until the vitamin effect had disappeared. One rat was left with the male for a period of 10 days and still gave the abnormal vaginal smear picture.

SUMMARY

Carotene in oil given daily for 15 days and representing as many as 3750 international units of vitamin A prevented directly or indirectly a normal vaginal smear picture.

Control rats that had received only the normal diet showed a normal oestrous cycle throughout the investigation.

Control rats given cottonseed oil and corn oil also exhibited a normal vaginal picture as observed by the smear technic.

Carotene in large amounts produced a rapid growth of the epithelium for a period of approximately 30 days. This was indicated by the large number of young nucleated epithelial cells which predominated in the vaginal smears.

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THE VITAMIN C CONTENT OF HUMAN MILK AND ITS VARIATION WITH DIET [1]

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The primary purpose of the present investigation was to find the approximate antiscorbutic value of human milk. There were important and closely related items which could be studied simultaneously, however, such as the range of variation in individual cases and the influence of wide variations in vitamin intake during short experimental periods. Of particular interest was the possibility thus afforded of establishing directly the quantity of vitamin C which could be considered normal for an infant.

EXPERIMENTAL

Method of analysis. The 2,6-dichlorophenol-indophenol titration technic was used, essentially as described by Bessey and King ('33). The method has been shown to give an accurate measure of the ascorbic acid content of most normal tissues of both plants and animals, and in an earlier investigation in our laboratory (Waugh and King, '33) it was found that the indophenol titration of cows' milk was accurate within the limits of biological assay. We found it unnecessary to precipitate the curd and filter before titrating, because the direct addition of 8 per cent trichloroacetic or acetic acid followed by dye titration gave satisfactory end points with a shorter time interval and less manipulation. Duplicate or triplicate analyses were made on 4 or 5 cc. portions of each

[1] Contribution no. 311 from the department of chemistry, University of Pittsburgh.

sample. Samples were obtained from one to three times per day and placed in refrigeration in aluminum capped test tubes whenever held during brief periods before titrating. The orange juice given as a vitamin supplement[2] was titrated regularly to provide an accurate record of the total vitamin intake provided in the special supplement.

Clinical records. The patients from whom samples were obtained had been under medical care long enough to give reasonable assurance of normal health. Their diets previous to hospitalization had apparently been representative for the community, and the hospital dietary was probably above the average in general quality. Most of the patients included in

TABLE 1

Vitamin C content of human milk (milligrams per cubic centimeter) and the effect of a special vitamin supplement

AVERAGE DAILY VITAMIN SUPPLEMENT	NUMBER OF CASES	AVERAGE AGE, YEARS	INITIAL VALUES 3 TO 6 DAYS	DAYS POST PARTUM					
				5	6	3	8	9	10
None	17	24.3	0.053	0.056	0.058	0.060	0.066	0.064	0.064
210 mg.	19	24.7	0.056	0.067	0.070	0.068	0.071	0.072	0.073
430 mg.	17	25.5	0.055	0.065	0.070	0.076	0.077	0.078	0.081

the study were given special supervision[3] to avoid dietary irregularities and to insure adequate care in recording the administration of orange juice and in obtaining samples. The cooperation of the patients was purely voluntary.

Experimental data and discussion. A summation of the major part of the investigation is given in table 1. It will be noted that the titration values for the three groups were comparable at the beginning of the test, and that there was a moderate but distinct rise in the antiscorbutic value of the milk from patients who received no special vitamin C supplement. This might be interpreted as a normal physiological

[2] We are indebted to the California Fruit Growers' Exchange for supplying the oranges as needed.

[3] The study was made possible by the generous cooperation of Dr. B. Harden, Miss Trumbull, superintendent, and other members of the staff of the Elizabeth Steele Magee Hospital, to whom the authors are greatly indebted.

change in the composition of the milk secreted, but a more careful evaluation of the data indicates that it represented the rise made possible in large part by the improved hospital dietary compared to the dietary before hospitalization. The number of instances where the titration values remained practically constant throughout the period of observation gave evidence that there would not have been such a distinct rise independent of increased dietary intake. The vitamin content of colostrum, based upon twenty cases, showed wide variation, but gave an average value that was comparable with normal milk. The slowness of obtaining samples during the first few days of lactation no doubt occasioned greater losses by atmospheric oxidation. For example, the average loss in titration value for eleven samples held in a refrigerator during 18 hours was 27.2 per cent. This was much greater than the loss observed previously for bottled market milk (Waugh and King, '33).

The relatively slow and limited rise in antiscorbutic value when patients received a special supplement of orange juice equivalent to 210 or 430 mg. of ascorbic acid per day is noteworthy in that it provides an indication of a maximum and approximately optimum level of secretion, above which an excessive dietary intake results chiefly in a rapid urinary excretion without disturbing the lactation level. It was not possible, with the limited facilities and personnel available to run regular and immediate titrations of urinary output. However, the titration data for four patients in the control group, five receiving a supplement of 165 mg. of vitamin per day, and ten receiving a supplement of 475 mg. of vitamin per day, showed eliminations of not less than 18, 48 and 296 mg. of vitamin per day respectively. These values are probably somewhat low due to unavoidable delays in titration. The oxidation loss is very rapid in alkaline urine. Similar rapid urinary excretion has been shown by Harris and Ray ('35) and a number of other investigators. Harris and Ray reported daily excretions of 11.7 to 38 mg. Marked differences in 'kidney thresholds' in relation to ascorbic acid excretion

probably account in large degree for the individual differences observed occasionally in the rate of depletion and protective requirement (both human and guinea pig).

There was a noticeable variation in the antiscorbutic value of the milk at different times of the day and from day to day, but the average difference between morning and afternoon samples was small. For 104 samples collected between 9 and 11 A.M., compared with the same number collected from the same patients on the same days between 3 and 6 P.M., the average values were 0.060 and 0.064 mg. per cubic centimeter respectively. The individual values for four patients living

TABLE 2

Increased vitamin content of milk, subsequent to receiving a special supplement of 500 cc. of orange juice (310 mg. of vitamin C) per day

PATIENT	INITIAL VALUE	SUCCESSIVE DAYS		
		1	2	3
	mg. per cc.	*mg. per cc.*	*mg. per cc.*	*mg. per cc.*
A	0.026	0.032	0.054	0.069
B	0.043	0.053	0.074	0.078
C	0.032	0.041	0.053	0.065
D	0.044	0.048	0.070	0.088
E	0.043	0.052	0.062	0.074
F	0.019	0.019	0.021	0.032

at home (average age of children, 43 days) and receiving representative dietaries were 0.062, 0.061, 0.055 and 0.053 mg. per cubic centimeter. The extremes of individual variation observed at any time were 0.0124 and 0.1081 mg. per cubic centimeter. The average value reported from thirteen cases by Harris and Ray ('35) during the course of this investigation was 0.056 mg. per cubic centimeter (lactation time was about 3 months).

In every case where the initial value was subnormal, and vitamin supplements were given over a period of 3 days or more, a distinct rise became evident. Table 2 illustrates the typical rapid response to a generous vitamin intake when initial values were distinctly subnormal. This appears to provide reasonably good evidence that the previous dietaries

had been inadequate for normal or optimum lactation in regard to this specific factor. The last patient listed in table 2 stated that she had eaten practically no citrus fruits or other noteworthy antiscorbutic foods, and that her major foodstuffs (before being admitted to the hospital) were rice and macaroni.

The normal level of vitamin C in human milk is strikingly higher than in cow's milk. Different investigators have reported varying results concerning the antiscorbutic value of cow's milk (associates of L. A. Rogers, '35; Riddell, Whitnah, Hughes and Lienhardt, '36; Sherman and Smith, '31), but a summary of the evidence would indicate that 25 to 30 cc. may be considered a minimum protective level for guinea pigs under optimum conditions of vitamin intake (spring pasturage). This would indicate an ascorbic acid content of approximately 0.02 to 0.025 mg. per cubic centimeter which is in good agreement with the titration data cited by Hughes and associates and Harris and Ray. Under general feeding and merchandizing conditions the average value for raw market milk is more nearly 0.010 mg. per cubic centimeter and secondary oxidation may decrease this readily to extremely low values. Since cattle are apparently capable of synthesizing all or the greater part of their vitamin C requirement (Thurston et al., '29), it is not surprising to find a lower lactation level. The general need for providing a special vitamin C supplement (i.e., in addition to cow's milk) for infant feeding is made evident by the above data.

It seems evident that the normal requirement of vitamin C per day for an infant may be estimated from the amount supplied in mothers' milk when the parent is receiving a good diet. On this basis, with a fluid intake of 21 ounces, the normal vitamin C supply would be approximately 40 mg. per day. In many cases, on a representative hospital diet without a special supplement, the intake would be 50 mg. per day. With increasing food consumption by the infant the normal intake would be correspondingly higher.

In the light of the above data concerning the low vitamin level of human milk when the mother's diet has not been adequate, and earlier data (Yavorsky, Almaden and King, '34) showing that occasionally infants are very deficient in vitamin C tissue reserves, the suggestion that infants and embryonic tissue generally may not need a dietary source of the vitamin (Rohmer et al., '35; Banerjee, '35) is very unfortunate. There is no clear-cut evidence to support the view that either guinea pig or human tissues can synthesize vitamin C from non-vitamin food material at any stage in their development.

TABLE 3

Relative constancy of vitamin C in milk when the initial lactation level was high, and when a special supplement was given

VITAMIN SUPPLEMENT	AVERAGE INITIAL LEVEL (3 TO 6 DAYS)	VITAMIN CONTENT ON SUCCESSIVE DAYS		
		1	2	3
mg. per day	*mg. per cc.*	*mg. per cc.*	*mg. per cc.*	*mg. per cc.*
165	0.081	0.083	0.083	0.081
250	0.075	0.075	0.071	0.073
375	0.080	0.081	0.081	0.082
375	0.083	0.082	0.085	0.087
500	0.072	0.083	0.087	0.088
500	0.072	0.080	0.083	0.083
500	0.073	0.071	0.078	0.077

The role of vitamin C in maintaining both resistance to infections (Clausen, '34) and protection against the injury of bacterial toxins (King and Menten, '35; Rinehart and Mettier, '34; Schultz et al., '35) indicates the importance of avoiding depletion of the tissue reserves. Without a special antiscorbutic supplement, the vitamin C reserves of formula-fed infants would be depleted steadily and an impairment of health would follow in corresponding degree.

SUMMARY

The vitamin C content of human milk was found to vary from 0.012 to 0.108 mg. per cubic centimeter, the average of fifty-three cases, 3 to 6 days post partum, being 0.055 mg. per cubic centimeter. On a good hospital dietary without

special supplements the average value rose gradually to 0.064 on the tenth day. The values for two groups receiving orange juice supplements equivalent to 210 and 430 mg. of vitamin C per day rose to 0.073 and 0.081 mg. per cubic centimeter respectively on the tenth day. When the mother was receiving an adequate diet the usual range of vitamin C was in the zone of 0.060 to 0.080 mg. per cubic centimeter. This indicates that the normal vitamin C intake for an infant is approximately 40 to 50 mg. per day during the first few weeks of life. Several cases with markedly subnormal antiscorbutic values were observed, and these rapidly approached normal when an orange juice supplement was given. Patients with initial high lactation levels indicative of a good state of nutrition showed relatively small increases when vitamin supplements were given. The excess quantities of vitamin were eliminated rapidly in the urine. The present investigation and data reported previously provide strong evidence against the suggestion that guinea pigs and humans can synthesize adequate quantities of ascorbic acid during gestation or infancy.

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LAFAYETTE BENEDICT MENDEL—AN APPRECIATION [1]

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In undertaking to speak of Professor Mendel and his work, I do so with a profound sense of humility, fully realizing that nothing I may say can add to the fame he has achieved, or deepen the esteem in which he is held by each of you. But despite this fact, I welcome the opportunity of paying my respects to one whom I have had the privilege of knowing intimately as teacher and friend.

Early in his career Lafayette Benedict Mendel demonstrated his unusual talents. In 1893, at the age of 21 years, he received the doctorate from Yale University. The following spring his thesis, published with Prof. Russell H. Chittenden, appeared in the Journal of Physiology. It is entitled, "On the Proteolysis of Crystallized Globulin." It deals with the separation and characterization of the proteoses and peptones produced by the action of pepsin-hydrochloric acid upon edestin. The paper is among the first to describe the enzymatic hydrolysis of a *crystalline* protein.

Since the publication of this thesis, more than 300 contributions have been made by Professor Mendel and his associates. Obviously, it will not be possible for me to review them in the time available. Furthermore, to do so would be quite

[1] This brief paper was prepared at the request of the Council of the American Institute of Nutrition, and was read at the annual meeting of the Institute in Washington, D. C., March 25, 1936.

It was not the purpose of the writer to give an account of the life and work of Professor Mendel. The intention was rather to emphasize the remarkable personal attributes of one who was a great leader and an inspiring teacher. The paper is published at the suggestion of the editorial board of the journal.

presumptious inasmuch as you are already familiar with their contents. However, it may be of interest, particularly to the younger members of this audience, to list some of the topics which he investigated, especially those which repeatedly attracted his attention. Of these, the following may be mentioned: The chemical changes during embryonic development; the alimentary processes, including the secretory activity of glands, the action of enzymes, and the paths of absorption of the digestive products; the formation and flow of lymph; the permeability of cell membranes; the regulation of blood volume; the metabolism of carbohydrates, proteins, fats, purines, pyrimidines, creatine, creatinine, and sulfur; the paths of excretion of inorganic salts; the rate of elimination of nitrogen under widely diversified conditions; the nutritive value of cereals and other natural food materials; and the utilization of purified carbohydrates and proteins. In collaboration with the late Dr. Thomas Burr Osborne, Professor Mendel published more than 100 papers upon growth, with particular reference to the importance of food 'accessory factors' and amino acids. His extensive investigations upon vitamins A and B, and the role of lysine, tryptophane, and cystine in growth are classics in the literature of nutrition.

An examination of Professor Mendel's contributions cannot fail to impress one with the intellectual resourcefulness of the man, and his remarkable versatility in research. Scarcely any aspect of nutrition escaped his scientific curiosity. Unusually gifted as a writer, he was as judicious in the preparation of his published reports as he was meticulous in the conduct of his laboratory investigations. The logical development of subject matter, clarity of expression, and forceful diction render his papers models of scientific literary perfection.

But it is not my purpose at this time to discuss Mendel the investigator and literary genius, but rather Mendel the teacher, the counselor, and the friend of students. These attributes possibly are less clearly appreciated by those who

have not worked under his direction. From year to year Professor Mendel attracted to his laboratory an unusually large number of young men and women. The departmental records, made available through the courtesy of Prof. Arthur H. Smith, show that ninety-two students received the degree of doctor of philosophy under his direction. In addition, 237 graduate students and ninety-six advanced research fellows and visitors received part of their training under his supervision.

Professor Mendel created a truly remarkable intellectual atmosphere in his laboratory. Though always kind and sympathetic in his relations with students, yet he had a ready faculty of impressing newcomers promptly with the conviction that their sole reason for being there was to accomplish something. An indefatigable worker himself, he rightly expected others to make full use of their opportunities. He had little time for the laggard, but exercised infinite patience and tact in dealing with those who were industrious and reasonably intelligent. During that period of his career with which I am most familiar—the period which the very recent graduates sometimes denote, to my chagrin, as the 'early days'—the professor practically lived with his students. He liked to watch them work, to observe how they went about their tasks; and each day, unless prevented by some administrative or other duty, he 'made his rounds,' as he said. His presence was always inspiring. During these frequent and informal conferences the students really came to know him, and to appreciate his unselfish spirit and whole hearted interest. If carelessness, lack of neatness in work, or poor technic was observed, it was immediately corrected; if encouragement was required, it was cheerfully given. With the beginner in research he emphasized the disciplinary value of unsuccessful experiments. Apparent failures never were to be taken too seriously, but rather were to be made use of in devising more satisfactory methods of attack. The relationship which existed between 'The Professor' and those working under his guidance is exemplified by the manner in which he invariably

referred to the group as his 'laboratory family.' As the years passed, and his keen intellect and mature judgment were more widely recognized and sought, his university, as well as innumerable outside committees and organizations, made greater and greater demands upon his time and energy. But despite the enormous responsibilities which rested upon him the welfare of his students was always uppermost in his mind.

Prefessor Mendel possessed an intimate and detailed comprehension of nutrition which was little short of astounding. He was always a prodigious reader. For this purpose, many evening hours, and at least one full afternoon per week were reserved. Any of his older students will recall how on Thursday afternoons he sat in the reading room of the Old Library completely absorbed in his work, and totally oblivious to everything about him. Early in his career he had established a regular habit of broadening his personal knowledge of the work of others. This became a lifelong practice. Once he remarked casually that while he was a student in Baumann's laboratory at Freiburg he utilized his 'spare time' by reading every paper in the first twenty-two volumes of Hoppe-Seyler's Zeitschrift. But even more remarkable than his capacity for reading, was his uncanny ability to remember what he had read. A personal experience will serve to illustrate this point. One day, in the course of his regular interviews with the graduate students, he came to my desk. After the usual inquiries as to the progress being made, I mentioned a difficulty encountered in the conduct of the investigation. The particular problem to which I referred has long since escaped my memory, but 'The Professor's' answer will never be forgotten. Without a moment's hesitation he said, "Oh, you will find a paper on that very subject in volume 17 of the Biochemische Zeitschrift." And then as he walked away he turned and added, "That paper begins about page 563." Needless to say, I was left gasping with astonishment. But the incident was not a unique one. Other students of his have had like experiences.

Much could be said regarding Professor Mendel's methods of class instruction. During the period to which I refer, the enrollment in his general course in physiological chemistry was relatively small, inasmuch as the medical students were taught as a separate group. The class periods were not divided into lecture and laboratory hours, and were used as conditions demanded. Lectures varied in length from a few minutes to 2 or more hours depending upon the importance and complexity of the subject under discussion. Quizzes, both oral and written, were given at frequent intervals, and usually without previous notice. Generally, part of the laboratory assignments were made at the beginning of the class period. 'The Professor' dictated the directions. Thus the romance of experimentation was not destroyed by the use of a laboratory manual containing the results to be expected in each manipulation. After a suitable interval, the students were called together for a conference in which their observations were checked and criticized. The climax of each period was 'The Professor's' summary of the day's accomplishments. In the pertinent and telling fashion of one thoroughly versed in the art of precise thinking, he emphasized the salient points and their applications. In the words of Macauley, "He had a wonderful talent for packing thought close, and rendering it portable." No student, save a moron, could experience one of those class periods without acquiring new enthusiasm for the subject in which he was being trained.

The high point of each week's activity was the seminar. It was then that the 'laboratory family' gathered to partake of a 2-hour intellectual feast planned and supervised by 'The Professor.' Only those of the immediate 'family' were permitted to sit at the table. An outer row of chairs provided places for the transients—that is to say, the visitors from other departments, and the out-of-town guests. Each member of the household was required to contribute to the 'meal.' From the more mature young men and women to the younger 'children' of 'the family,' the contributions were carefully adapted to the 'digestive' capacities of the individuals. Correct table technic was expected, and few reprimands were

necessary; but woe unto him who spoiled the 'meal' by inadequate preparation! The kindly figure at the head of the table *could* become exceedingly stern. The mimeographed programs constituted the menus. Occasionally they appeared in English or French, but more frequently in German. Sometimes they provided historical 'food for thought,' as represented by the contributions of a single distinguished investigator, or the development of an important theory. Perhaps more generally they were formulated from the 'articles of diet' in the current literature. Whatever their composition they were always well balanced, and capable of ready assimilation, if properly prepared.

On these weekly occasions Professor Mendel was at his best. He literally radiated enthusiasm. By skillfully directing the discussion of the students, and by his own well chosen remarks, he placed each contribution in its proper setting with respect to the subject as a whole. His wide personal acquaintance with the leading investigators abroad enabled him frequently to relate some interesting or amusing incident about the authors whose papers were being discussed. He never failed to see, or make the most of, the humor in any situation. I recall vividly an occasion of this sort. A paper by a distinguished German physiologist had just been presented by a member of the class. The author's name brought to Professor Mendel's mind an incident which occurred while he was a student in Germany. It seems that the professor of physiology was demonstrating to his class the effect of stimulating a certain nerve. The nerve was small and rather difficult to locate. Although he had performed the experiment many times before, he was experiencing considerable embarrassment on this particular day, for the nerve actually seemed to be missing. The situation was becoming tense. Suddenly his Diener had an inspiration, and in a tone loud enough for the students to hear remarked, "Herr Geheimrat, da is der Nerv!" "Unsinn," said the Geheimrat, "Was wissen Sie von dem Nerv?" "Ja, ja," said the Diener, "Sie haben recht, Herr Geheimrat; aber da is der Nerv." Thus the great

physiologist was humiliated by his Diener. But this story is not the one I wish to emphasize. The sequel is the important matter. After relating the incident, Professor Mendel, with a merry twinkle in his eyes and a faint suggestion of a smile remarked, "You know, one German Diener is more help in a laboratory than are two American assistants." An indelible impression was made on my mind; and well might it, for at that very time I happened to have been 'The Professor's' laboratory assistant.

To his students Professor Mendel was more than a distinguished scientist and a great teacher. Somehow, he directed the aspirations and broadened the perspective of those who came under the charm of his personality. He was not content merely to impart facts to, or to perfect the scientific technic of those about him. These things he did; but in addition, he implanted ideals—ideals of tolerance, unselfishness, intellectual honesty and service. Helmholtz, in speaking of his noted teacher, Johannes Müller, said, "Whoever comes into contact with men of the first rank has an altered scale of values in life." No more appropriate tribute than this could be paid to our distinguished friend whom we honor on this occasion. His students saw him as the personification of the ideals which they admired. They caught his spirit; and determined, perhaps unconsciously, to 'carry on' in his way. Because of these attributes so difficult to describe, and yet none the less real, Mendel won and retained the confidence, the respect, and the devotion of his pupils. He became their guide and counselor. Recently, one of his former students wrote, "It is our task to continue his ways of doing things, for we have learned their value." Another said, "It will be difficult for some of us to plunge ahead without his encouragement." And still another remarked, "I think those of us who knew him longest will miss him most." As we contemplate Mendel's achievements, and evaluate anew his great contributions to the science of nutrition, we may anticipate with confidence that his work will continue in the lives of the multitude of young men and women whom he trained and inspired.

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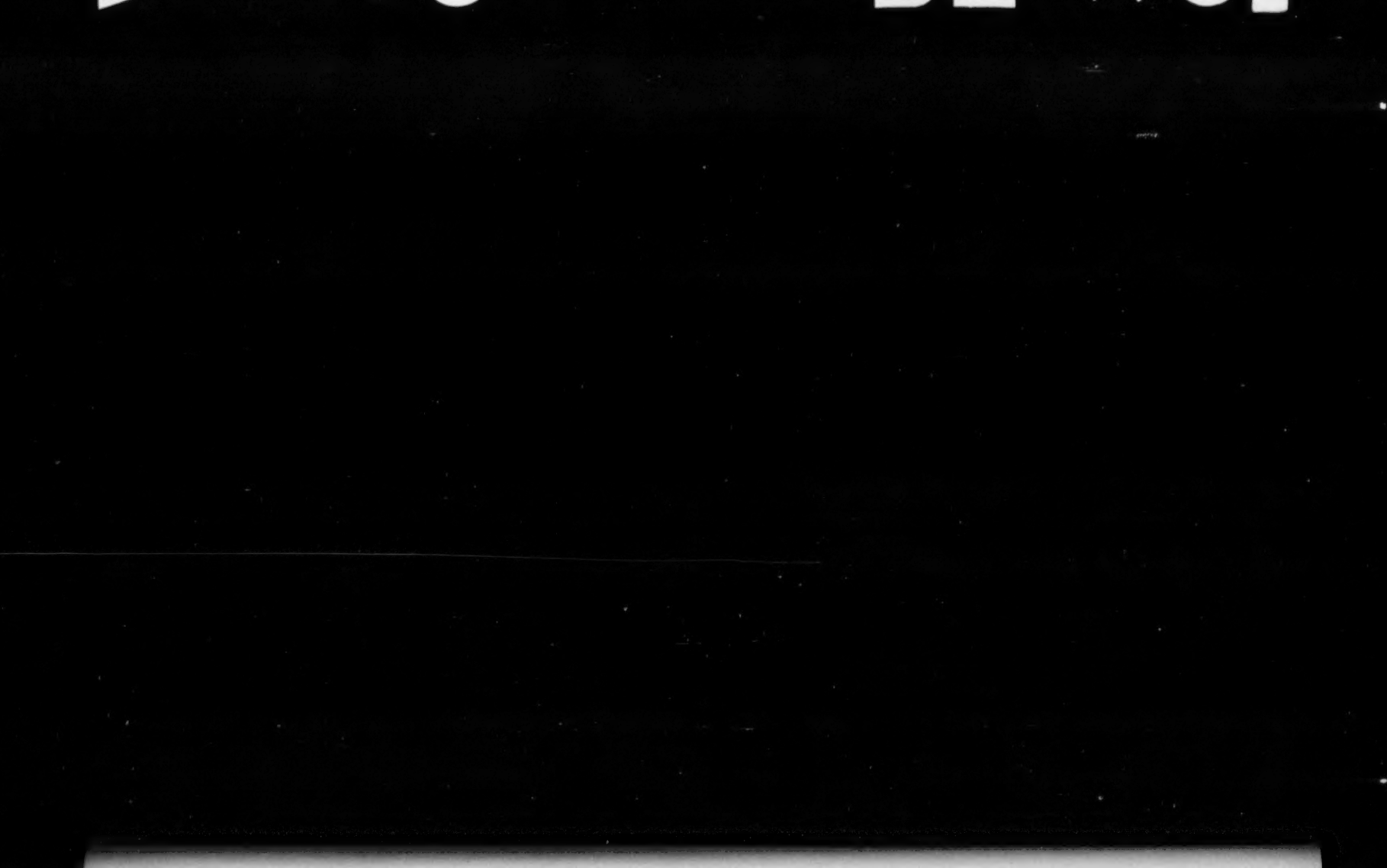
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